THE UNIVERSITY OF ALBERTA

THE THYMUS AND ERYTHROPOIESIS

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled THE THYMUS AND ERYTHROPOIESIS submitted by John Harry Chritchley in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Certain human diseases as well as a number of experimental studies suggest that the thymus might have an effect upon erythropoiesis. The results of these studies are for the most part inconclusive and the influence of the thymus upon erythropoiesis is still speculative.

A series of experiments was designed to measure the post irradiation erythropoietic recovery and erythropoietic spleen colonies in thymectomized mice. Thymectomy was found to have no effect upon erythropoietic recovery in irradiated mice though recovery was studied over a wide range of irradiation doses. Neither anemia nor the degree of immune competence of the experimental mice accounted for the failure to demonstrate a thymic influence upon erythropoiesis.

An attempt was made to influence the path of differentiation of a stem cell hypothesized to be common to both the erythropoietic and lymphopoietic lines. Neither bleeding nor an immune response could be shown to exert a competitive demand upon the progenitor stem cell.

Some of the studies indicated that the thymus might influence the development of non-erythropoietic colonies and that post irradiation bleeding of normal mice might affect the numbers of non-erythropoietic spleen colonies.

It was found that the anemia which results from 550 r induces maximal erythropoietic stimulation such that bleeding is unable to further stimulate erythropoiesis.

Bone marrow taken from mice which had been bled was found to

be deficient in its ability to restore splenic erythropoiesis, spleen weight, and probably spleen colonies when grafted into irradiated hosts, but neither bleeding nor antigen challenge to a donor mouse affected the ability of its bone marrow to restore erythropoiesis in the bone marrow of a radiated host. Antigen did increase the numbers of bone marrow colony forming units and the colonies which they formed were probably not erythropoietic.

In order to perform these studies, techniques were devised to thymectomize and to bleed mice. These are described.

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1. THE ROLE OF THE THYMUS:

The role of the thymus in the immune response has been intensively studied since 1961 when Miller (1) reported that thymectomy was associated with certain deficiencies in immune responsiveness. Despite considerable interest and investigation into the relationship between the thymus and the other cellular elements produced by the bone marrow, its influence upon the rest of the hemopoietic system, if any, is still speculative.

Miller found that thymectomized newborn mice had a deficiency in the lymphocyte population of their blood and lymphoid tissues, an inability to reject foreign skin grafts, and that they suffered premature death, which he attributed to infection. Since then it has been established (2) that cell mediated immune responses such as graft rejection and delayed hypersensitivity reactions are under thymic control. Full immune competence does not, therefore, develop in a neonatally thymectomized mammal, but it is not immediately lost in thymectomized adults. This is because immune competence depends upon antigen-reactive cells (ARC) and antibody-forming cells (AFC), which once produced continue to function efficiently for a period in the thymectomized animal (3). Furthermore, there is a circulating pool of thymus derived lymphocytes (4), some of which are long-lived [months in rodents (5), and possibly years in man (6)]. Total body irradiation administered to a thymectomized adult does impair the cell-mediated immune response (7), probably by

depleting numbers of ARC, AFC, and circulating lymphocytes.

2. THE DEPENDENCE OF THE THYMUS UPON THE BONE MARROW:

The precursor cells of the immune response are, however, not thymus derived, but are bone marrow derived cells (3) (4). Indeed, the whole of the lymphatic system which includes the thymus, is believed to be initially and continuously populated by cells of bone marrow origin (2) (8). There is evidence which indicates that the thymus must receive bone marrow stem cells in order to achieve normal embryonic structural development (9). It then contributes lymphocytes to the spleen, lymph nodes, circulating blood, and thoracic duct (2) (4).

It is generally agreed (10) that the thymus exerts an inductive influence upon precursor cells of hemopoietic origin, which results in expansion of the thymus-dependent lymphoid cell population, as well as lymphoid differentiation and proliferation to form immuno-competent cells.

Whether the thymic contribution is humoral, cellular, or a combination of the two, has been discussed by a number of authors (2) (11) (12) (13), but this question is still unresolved.

Despite its obvious importance, little is known about the hemopoietic precursor cell. It is not clear whether there are separate stem cells for both the myeloid and the thymus-dependent cell pools; however, the colony forming cell (CFU), which is a stem cell capable of forming clones of erythropoietic, myelopoietic, and megakaryopoietic lines, or its precursor, possibly functions as a stem cell for the lymphocytic line (14) (15) (Page 14) as well. Precisely such an opinion is expressed by Makinodan and Albright (16). They support the view that

the cell which is initially involved in the antibody response to antigen (X or PC_1 cell) is comparatively uncommitted and closely related to the pluripotential CFU.

3. EVIDENCE RELATING THE THYMUS TO ERYTHROPOIESIS:

As long ago as 1928, Preisel (17) noted a clinical association between possible thymus dysfunction and erythropoiesis when he reported a patient with thymoma and pure erythroblastic hypoplasia. It was later estimated that five to six percent of people with thymoma have pure red cell anaemia (18), and what is probably more significant, that fifty percent of adults with pure red cell anaemia have thymoma (19). These findings suggest that the thymus may influence erythropoiesis. Experimental proof of such an influence would be of immunological and hematologic interest. A review of the pertinent clinical and experimental studies which relate the thymus to erythropoiesis, indicates that a possible thymic role upon erythropoiesis is still controversial.

Lists and reviews of cases of pure red cell anaemia associated with thymoma have appeared regularly in the medical literature (20) (21) (22) (23). None of the authors have drawn any firm conclusions regarding a cause and effect relationship such as competition for a common stem cell, or absence of some erythropoietic stimulating function which might be present in, or produced by the normal thymus. Barnes (22) suggested that a pathological thymus might inhibit erythropoiesis through production of some humoral factor, possibly antibody. Dameshek et al. (21) in a review of the subject, questioned whether the alteration of

thymus function and development of a thymoma might be the end result of another abnormality which simultaneously results in pure red cell hypoplasia.

Other clinical observations have indicated that the thymus is a site of red blood cell production. Gilmour (24) referred to transient thymic erythropoiesis in the human fetus, and noted that it was limited to the intralobular septa or perithymic tissue. This was paralleled by the discovery by Albert et al. (25), that there were erythroblasts in thymuses of autopsied human fetuses, infants, and young children.

The latter group of investigators was subsequently able to demonstrate erythroblasts in the thymuses of normal four-day to twelve-week old mice of different strains (26) (27). To test the erythropoietic properties of the thymus, they enclosed fresh adult murine thymus cell suspensions in millipore diffusion chambers and cultured them in the abdominal cavity of isogeneic mice. They found a seventy-nine percent increase in erythrocytes in the chamber over a five-day period, as compared with viable spleen cell suspensions similarily cultured (28). They conclude that many of the cells in the thymus are erroneously identified as lymphocytes, and that up to eighteen percent of thymus cells have an erythropoietic function, and it is thus an important site of erythropoiesis. Since the bone marrow from comparable mice was normal, they rejected the possibility that they were observing a phenomenon due to extramedullary hematopoiesis.

Saki and Shirasawa (29), claim to have induced hematopoiesis in the thymuses of four-month old rabbits. This was accomplished by

injecting microsomes from the thymus of normal or sensitized rabbits directly into the thymus of an homologous rabbit. They are of the opinion that some thymocytes are stem cells which can develop into myeloid cells.

Other investigators, like Campbell et al. (30), have been unable to demonstrate any evidence for either erythropoietic activity in the mouse thymus, or any erythropoietic function of the mouse thymus. This group studied erythropoiesis in the thymus, spleen, and bone marrow of the adult mouse. They assessed erythropoiesis morphologically with hemoglobin stains, and functionally by radioiron uptake and autoradiography. They studied normal, thymectomized, and sham thymectomized mice, which were subjected either to sublethal irradiation, or lethal irradiation followed by bone marrow or thymus cell grafts.

Metcalf (31) measured red cell count, hematocrit, hemoglobin, reticulocyte levels, osmotic fragility, and red blood cell morphology in thymectomized and sham thymectomized mice. He bled, or injected cortisone acetate into groups ranging in age from neonates to five weeks old. In addition, he studied mice which were grafted with multiple subcutaneous thymus grafts from one-day old isologous donors. Though he followed the mice for up to three months, he was unable to demonstrate any significant erythropoietic differences between thymectomized, thymus grafted, and control mice.

The above studies of Campbell et al., and Metcalf, cast doubt both on the tenet that the thymus participates in erythropoiesis or is in any way able to influence total body effective erythropoiesis. That

thymus cell infusions fail to promote erythropoietic recovery in irradiated mice, is further documented by Goodman and Shinpock (32), and others (33). Goodman and Shinpock did observe, however, that a combination of parental thymus, and bone marrow cells produced a six to eight fold increase in the erythropoiesis of the grafted marrow in the lethally irradiated F_1 hybrid, as determined from radioiron uptake in the recipient's blood and spleen.

The spleen is a prominent organ of the reticuloendothelial system and as such is commonly used as a source of cells for immunological assay (Page 37). In the mouse it is also an important organ of erythropoiesis (34) (35) (36), and is functionally and ferrokinetically analogous to the bone marrow (37). In addition, bone marrow stem cell potential can be measured by spleen colony formation (Page 10). Some investigators have reported a thymic influence on splenic erythropoiesis which parallels the study of Goodman and Shinpock. As early as 1960, Metcalf (38) noted that thymectomizing an adult mouse decreased not only the follicle size and spleen weight, but also the size of the splenic red pulp (erythropoietic area). Auerbach (39) observed that mouse thymus fragments cultured in vitro with embryonic mouse spleen fragments, increased the growth of both the erythropoietic and lymphopoietic elements of the spleen.

The studies above, suggest that the thymus in some way promotes splenic erythropoiesis, however, other investigators have obtained evidence suggesting that the contrary is true. Miller (40) found that thymectomized opossum embryos had an abnormal persistence, and even increase in spleen and lymph node myeloid cells, mostly erythroblasts. There were,

in addition, increased numbers of immature to mature erythroblasts, megaloblastoid-like erythroblasts, and other features which suggested maturation arrest and ineffective erythropoiesis. Normal and partially thymectomized pouch embryo litter mates showed no alterations in hematopoietic tissues. Similar changes have been reported in the bone marrow of rats thymectomized two days after birth (41). Miller's group theorized that the thymus might suppress myeloid tissue. These studies were performed on immature animals, but Globerson and Feldman (13) have since reported increased splenic erythropoiesis in thymectomized adult mice. They subjected thymectomized and intact C3H and C57BL mice to sublethal total body irradiation, and observed that the erythrocytic spleen colonies (Page 10) appeared earlier and persisted considerably longer in the thymectomized group. Further, they observed that by grafting thymuses into the experimental group, that splenic erythropoiesis declined as splenic lymphopoiesis increased. They argue that the main effect of thymectomy in irradiated mice is the prevention of regeneration of lymphocytic cells, and though they draw no conclusion as to the mechanism of erythropoietic influence, their discussion implies a permissive effect in contrast to Miller's suggested suppressive effect.

It must be considered that the thymectomized neonatal mammal or irradiated thymectomized adult mammal is in a sense an immunological cripple, defenseless against a variety of antigens, including some microorganisms. The thymus may not be directly involved in immunity to tumor virus, like Friend disease Virus (FDV) (42). The FDV is implicated, however, in the etiology of polycythaemia, and it has been shown that

mice infected with this virus undergo a marked suppression of both their cellular and humoral immune capacity, which Ceglowski et al. believe occurs at the stem cell level (43). The possibility remains therefore, that other undiscovered agents might influence erythropoiesis in a thymectomized susceptible animal, or indeed compete for a precursor cell common to both the immunologic and erythrocytic systems.

Finally, the wasting disease which develops in neonatally thymectomized mice has certain characteristics of autoimmune phenomena, and associated hematologic alterations have been described. Yunis et al. (44) recently noted that Coombs positive haemolytic anaemia and extramedullary (especially splenic) hematopoiesis were common in A and C57BL/Ks strains of mice during the post thymectomy wasting period. These hematologic alterations were, however, uncommon in neonatal C3H mice, despite the observation that ninety-eight percent of them developed wasting disease-post-thymectomy.

Though autoimmune phenomena may account for some of the conflicting observations discussed, others may arise from difficulties in morphological identification of precursor cell lines, and still others may result from unrecognized shifts in total body erythropoiesis as has been described in the graft versus host reaction (45).

The studies which follow are an attempt to evaluate the effect of the thymus upon erythropoiesis in the adult mouse. In order to avoid the pitfalls mentioned above, the following precautions have been taken. Firstly, all mice used are C3H, in order to minimize any hematologic alterations which might arise from autoimmune phenomenon. Only

male mice were used, to eliminate any variation which might occur as a result of the estrus cycle, and where possible, in any one experiment, mice of the same age were used, to eliminate any affect of age. Secondly, erythropoiesis in the bone marrow, spleen, and circulating red blood cells is measured by its ability to take up radioactive iron, and it is further measured by both the hematocrit and reticulocyte count. The radioiron uptake should also identify any major shifts of erythropoiesis. Thirdly, to minimize any effects of anaesthesia and surgery, the control group is subjected to a nearly identical sham operation. Fourthly, erythropoiesis is measured in a post-irradiation recovery period, since it is reasonable to expect that any thymic influence might be magnified in a recovering bone marrow. Finally, hemopoietic spleen colonies are counted concurrently with the other studies. A discussion of the significance of spleen colonies follows.

4. THE COLONY FORMING UNIT ASSAY AND ITS SIGNIFICANCE:

Spleen colonies are discrete, round, or oval nodules of proliferating hemopoietic cells which are visible on the surface of a spleen, and their numbers are used as an assay of hemopoietic stem cells, commonly referred to as colony forming units (CFU). Evidence to be described, indicates that a CFU is a stem cell for hemopoietic cell lines, and the precursor of the CFU is a stem cell also for lymphopoietic cell lines. Any influence which the thymus has on hemopoiesis might, therefore, be at the level of this precursor stem cell.

Numerous investigators, many examining the proliferative

potential of bone marrow or the influences of various chemotherapeutic agents upon the hemopoietic system, have used this assay, and a considerable amount of information about the nature and behavior of spleen colonies and CFU has been accumulated since 1961.

5. THE FORMATION OF SPLEEN COLONIES:

Till and McCulloch (46) first produced spleen colonies by injecting mouse bone marrow cells intravenously into a lethally irradiated isologous host. The number of resulting colonies proved to be dependent upon the numbers of viable stem cells within the inoculum, and the numbers of these which arrived at and proliferated within the spleen. Injections of whole blood (47) (48), the buffy coat of peripheral blood (49), cells from the peritoneal cavity (50), and cells from normal fetal liver (51) have all been shown to produce spleen colonies. Neither thymus cells, nor lymph node cells are capable of forming spleen colonies, even after phytohemagglutinin stimulation, except when the cells are given in such large doses that contaminating CFU from the circulating blood would be expected (52). Colonies produced by injection are referred to as exogenous, and their numbers decline exponentially as the irradiation dose to the inoculum graft increases. Furthermore, the relationship between the mean number of nucleated marrow cells injected and the number of colonies per spleen may be depicted graphically as a straight line which passes through the origin (46).

Spleen colonies identical in appearance and behavior are formed in a less heavily irradiated mouse without the injection of cells (53) (54).

These endogenous colonies develop from surviving CFU in the mouse, and their number also decreases as the irradiation dose increases.

As a measure of bone marrow regenerative potential the endogenous colony technique is as accurate as the exogenous colony technique (55).

6. THE CLONAL NATURE OF SPLEEN COLONIES:

The dose response studies of Till and McCulloch cited above, suggested that for both the exogenous (46) and endogenous (55) assay, one spleen colony is derived from the proliferation of a single cell. Evidence in support of this clonal theory has come from studies of chromosomally labelled cells. Becker et al. (56) irradiated recipient mice both before and after injection of bone marrow cells, and found that most of the mitotic cells in an individual colony had the same uniquely abnormal karyotype. Chen and Schooley (57) produced radiation chimeras bearing three distinct kinds of chromosomally labelled cells when they injected a bone marrow and spleen cell suspension which contained equal numbers of CBA/H, CBA/H-T_6T_6, and T_6/CBA cells, into a lethally irradiated CBA/H mouse (primary host). Bone marrow cells from the primary host were subsequently injected into a CBA/H secondary host, and 41 of 48 spleen colonies produced contained cells of one karyotype only, whilst the other seven colonies had one to four cells of host karyotype within them. All three karyotypes were observed within the colonies of one spleen. Wu et al. (58) prepared bone marrow cell suspensions in which some of the cells had abnormal radiation-induced chromosomal markers.

In each case where a colony contained this marker, more than 90 percent of the dividing cells in the colony also contained it. The study of Barnes et al. (59), in which more than 4000 mitotic cells from 309 colonies, many with marker chromosomes, were counted, led them to conclude that a spleen colony is the product of the proliferation of a single cell.

7. THE PLURIPOTENTIAL NATURE OF THE CFU:

In their original report (46), Till and McCulloch noted that individual spleen colonies could contain cells of granulopoietic erythropoietic and megakaryopoietic lines, implying that the CFU was multipotential. Using the chromosomal marker technique, they were subsequently able to show that there were, in one colony, cells which took up Fe^{59} characteristic of erythropoietic lines, and other cells which possessed peroxidase-positive granules characteristic of granulopoietic lines, and both lines contained the same aberrant karyotype (58). Nevertheless, for the first week of their development it has been shown that most spleen colonies are composed of a single differentiated cell line (56).

Curry and Trenton (60) examined the cellular imprints of large numbers of exogenous spleen colonies of different ages, and the following generalizations regarding the cellular composition of spleen colonies can be assumed from their study:

i. From four to nine days, colonies are composed of a single differentiated hematopoietic cell line, together

with undifferentiated cells.

ii. Erythropoietic colonies appear early, grow rapidly, and are the predominant colony type. By ten days a typical exogenous erythropoietic colony contains about a million viable cells. Granulopoietic elements are usually not identified before day eight.

iii. Neutrophilic colonies are the second most common type. They grow less rapidly and more diffusely, and are therefore less obvious grossly. The ten day exogenous neutrophilic colony has only an average of a third of a million cells, and unlike erythroid colonies which are found exclusively in the red pulp, neutrophilic colonies occur often along spleen trabeculae or in subcapsular sheets.

iv. In addition to megakaryocyte colonies which are less common than erythroid or granulocytic colonies, pure eosinophilic colonies are found but are extremely rare and small. Mast cell colonies were not seen.

v. Mixed colonies have the highest cell count by day ten, are predominantly erythroid, and increase in size at the expense of the pure colonies.

It has since been shown that ten days post irradiation, 75 percent of endogenous colonies are pure erythroid, and 25 percent mixed (61).

Wolf and Trentin (62) transfused cell suspensions made from pure erythroid colonies into a secondary irradiated host and found this

produced both erythropoietic and granulopoietic spleen colonies in the same ratio as they were present in the primary host. They concluded that spleen colonies contain multipotential or monopotential or both types of CFU.

The evidence presented, therefore, implies not only that a CFU is probably a single, multipotential cell which may differentiate into more than one mature form, but since it gives rise to further CFU, it has the characteristics of a true stem cell.

8. THE RELATIONSHIP OF THE LYMPHOID CELL TO THE CFU:

Curry and Trenton were unable to find evidence of lymphoid colonies in the spleen (60). They concluded that the regeneration of lymphoid cells occurs not by nodules but by a gradual diffuse replenishment of the spleen lymphoid follicles. This replenishment is not morphologically obvious until twelve days post transfusion, at which time erythropoietic colonies are nearly confluent.

The study by Wu et al. (14), in which they used the two stage technique, already described, to produce spleen colonies labelled by the presence of an abnormal karyotype, has resulted in strong supporting evidence that the lymphoid cell population is related to the CFU. They found that bone marrow, taken from mice in which thymus alone, or if enough time has elapsed, both the thymus and the lymph nodes had been replaced by lymphocytes containing an abnormal karyotype, produced exogenous spleen colonies of the identical karyotype. They were unable to prove that any of the labelled lymphocytes were antibody-producing

cells, however, more than fifty percent of the dividing cells in their antibody-forming cell assay contained the abnormal chromosomes.

They concluded that either lymphocytes are descended from CFU, or that both cells have a common progenitor. Edwards and Phillips (15) have recently provided evidence which indicates that rosette-forming cells, which are single cells carrying specific antibody at their surface, are also derived from the same hemopoietic precursor cells which form spleen colonies. Their study which involved the repopulation of lethally irradiated mice with bone marrow containing unique chromosomal aberrations, does not prove whether the hemopoietic precursor cell is a CFU or its precursor.

Lymphopoietic cell lines and cells capable of exhibiting properties of an immune response in vitro (rosette-forming cells) are therefore related to the other cell lines of the hemopoietic system by common ancestory. It is possible then, that the thymus, which plays an important role in lymphoid cell proliferation, differentiation, and immune competence, might influence erythropoiesis at the level of the common ancestor. If this is the case, then ablation of thymic function might be demonstrable through increased numbers of spleen colonies. This result would both support the findings of, and delineate the mechanism by which thymectomized mice produced more erythropoietic nodules and had inappropriate erythropoiesis. On the other hand, if the thymus is in any way necessary for the transport of CFU or development of spleen colonies, then thymectomy might result in reduced numbers of the colonies. This finding would support the findings of, and delineate the mechanism

through which thymus cells might hinder erythropoiesis.

The following studies are designed, therefore, to examine both post radiation erythropoietic recovery, and spleen colony formation in thymectomized and sham thymectomized adult mice.

II. MATERIALS

1. ANIMALS:

The experimental animals were C3H/HeJ mice, obtained at four to six weeks of age from the Jackson Laboratory[1]. Except for one experiment which included ten females (Page 68), only male mice were used.

The mice were housed in plastic or fiberglass cages with wood shaving nesting material, and kept in a temperature-controlled (72 $\pm$ 1°F), ventilated room prior to, and during the experiments. During experiments, mice were caged in groups of three or less, and the room was constantly illuminated by fluorescent lights. Vit-a-mite cubes[2] (a vitamin-enriched blend of fishmeal, bonemeal, wheat, and oats), and tap water were supplied to the animals ad libitum.

2. IRRADIATION:

Mice to be irradiated were placed in perforated plexiglass cages measuring 20 x 20 x 5 cm. with the lid in place. The cages were separated into three equal compartments which held up to five mice each. Initially, mice were individually separated by cardboard barriers, but this was discontinued when it was observed that they tended not to crawl over one another when the lid was secured. Groups of eleven to fifteen mice were irradiated at one time.

Irradiation was performed on a Picker Vanguard, 270 K.V. X-ray machine with a Half Value layer (HVL) of 1.5 mm. Cu., and filtration of 0.2 mm. of Sn. 90.9 r per minute was delivered at 50 cm. through a 20 x 20 cm. port.

Since the f rad/roentgen (muscle) equalled 0.96, the absorbed dose (rads) at the mid-plane of the cage (52.5 cm.) after correction for backscatter was approximately equal to the delivered dose.

3. Fe^{59}:

Radioiron was obtained as Ferric Chloride, $Fe^{59}Cl_3$ [3]. This solution was buffered to a ph of 1.5 - 2.5 with HCl or NaOH, and preserved with 0.9% benzyl alcohol. The dose was based upon the specific activity calculated for twelve o'clock noon on the day of the experiment. Fe^{59} has a half life of forty-five days.

4. ANAESTHETIC:

Sodium pentobarbital (Diambutal)[4] was given by intra-peritoneal injection. Each ml. of the commercial product contained sodium pentobarbital 60 mgm., alcohol 10%, propylene glycol 20% in water, and the ph was adjusted with NaOH. Five ml. was diluted to 40 ml. with 0.85% NaCl solution, resulting in a solution with a concentration of 7.5 mgm. of sodium pentobarbital per ml.

One ml. (0.6 mgm.) atropine sulphate was diluted to six ml., producing a concentration of 0.1 mgm/ml.

Animals were injected intra-peritoneally with 0.1 ml. (0.75 mgm.) sodium pentobarbital per ten grams body weight, and 0.1 ml. (0.01 mgm.) atropine sulphate.

Tuberculin syringes calibrated to 0.01 ml. were used for all injections.

Using this regime, good surgical anaesthesia was achieved within ten minutes and persisted up to one hour. No deaths were attributed to

the anaesthetic. The atropine was used to reduce tracheobronchial secretions.

The cut end of the tail from which blood was taken for either reticulocyte counting or the bleeding study, was cauterized with an electrodesiccation apparatus (Hyfrecator)[5].

Thymectomy, sham thymectomy, and all intravenous injections were performed under a lens[6] which magnified the object about 1.7 times.

Blood for micro-hematocrit or reticulocyte determination was drawn into heparinized capillary tubes (Red Tip)[7] which were sealed for hematocrit determination with critocaps[7].

FOOTNOTES

[1] The Jackson Laboratory, Bar Harbor, Maine, U.S.A.

[2] North West Feeds, Edmonton, Alberta

[3] Abbott Laboratories, North Chicago, Illinois, U.S.A.

[4] Diamond Laboratories Incorporated, Des Moines, Iowa, U.S.A.

[5] Birtcher Corporation, Los Angeles, California, U.S.A.

[6] Magnifier, Luxo Lamp Canada Limited, Ste. Therese, Quebec

[7] Fisher Scientific Company, Edmonton, Alberta

III. METHODS

1. THYMECTOMY:

Thymectomy was initially performed by scissor dissection with animals pinned by the paws to a dissecting board. This technique resulted in a high mortality from either bilateral pneumothorax or rupture of the great vessels, and increased the risk of post operative infection because of the wounds in the paws. Incomplete thymectomy was common in the survivors.

A satisfactory method was adopted and simplified from one described by Sjodin et al. (63). Following light ether anaesthesia, mice were anaesthetized with an intra-peritoneal sodium pentothal and atropine sulphate mixture. The fur on the anterior chest wall from xiphoid to mandible between the anterior axillary lines was removed with an electric hair clipper. The mouse was placed supine on a sloping dissecting board with the head at the raised end which was turned toward the operator, (Fig. 1). Two elastic bands kept the mouse immobilized. The dorsiflexed neck kept the airway open and elevated the thymus in the thorax, thus eliminating the need for abdominal pressure. The surgical field was cleaned, as were all the instruments, in 70% isopropyl alcohol. The skin and superficial fascia were cut longitudinally with scissors from the mandible to the fifth rib along the midline. A suture at least 5 cm. long was tied into the caudal end of the incision to facilitate rapid closure after thymectomy. The submaxillary salivary glands and sternohyoid muscles were separated by scissor point retraction, and

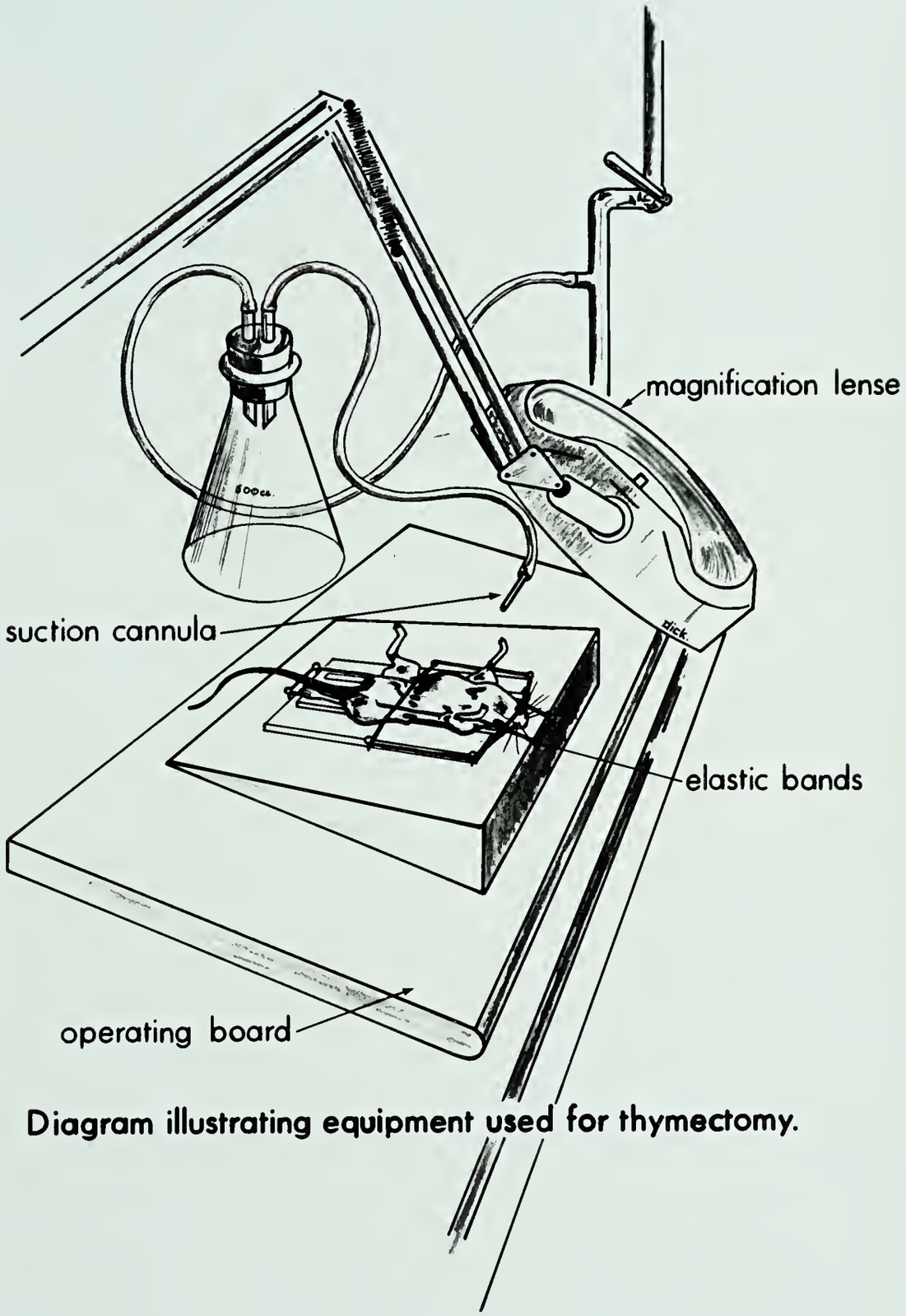


Diagram illustrating equipment used for thymectomy.

the sternum split to the fourth rib with scissors. A glass suction cannula, which had been made by heating 7 mm. glass tubing and drawing it out to a 2 mm. diameter, fire-polished base, was applied immediately to one or both thymic lobes. Traction on the cannula while retracting the left side of the rib cage laterally, separated the thymus from its attachment, and allowed it to be aspirated into the suction tube where it could be visualized, and the completeness of thymectomy assessed. The skin edges were approximated with one continuous silk suture. The procedure was carried out under the illuminated magnifying lens. Rarely was there any bleeding or respiratory distress. Any animal which bled during the surgery or later, from the skin edges, was eliminated from the study.

Sham thymectomy was identical except that the thymus, after being gripped by the suction cannula, was permitted to fall back into the thorax.

Post-operatively, the mice were placed prone on fresh wood shavings under a warming lamp.

2. RETICULOCYTE COUNTS:

Reticulocyte counts were performed two days after surgery on the first seventy-three animals, to see if the surgery had stimulated erythropoiesis. Under light ether anaesthesia the tip of the tail was amputated and a heparinized capillary tube half filled with blood. The blood was blown gently into an equal volume of New methylene blue, on a clean glass slide. The mixture was drawn back into the pipette, which was placed in a horizontal position for at least six minutes before being remixed and smeared on each of two slides. Two independent examiners counted 1,000

cells on each slide with the aid of the grid of a Miller Ocular micrometer disc (64). The mean reticulocyte count was 1.6% with a range of 0.9 to 2.4%. The reported normal mean is 2.8% (65). It was unnecessary to exclude any animals from the study on the basis of the post surgical reticulocyte count, and this procedure was discontinued.

3. INTRAVENOUS INJECTIONS:

A 30 gauge needle was used for all radiciron injections, and a 26 gauge needle for injections of cells. Needles were fitted to a 1 ml. tuberculin syringe calibrated into 0.01 ml. increments. All injections were into the lateral tail vein. In order to dilate the veins mice were placed either in a plexiglass irradiation cage warmed by the beam of a desk lamp, or in a 37°C incubator for ten to fifteen minutes. They were transferred to a plexiglass restraining cage for the injection. The tails of mice which received bone marrow injections were swabed with 70% isopropyl alcohol prior to injection.

4. PREPARATION OF Fe^{59} TRANSFERRIN COMPLEX:

Radioactive iron for intravenous injection was bound *in vitro* to transferrin. This was achieved by incubating fresh or fresh frozen normal mouse serum with $Fe^{59}Cl_3$ for one hour with occasional agitation, in a 37°C incubator. The concentration of Fe^{59} for ferrokinetic studies was 2.5 μc/ml. The dose injected was 0.5 μc Fe^{59}/20 gm. body weight in a volume of 0.20 - 0.30 ml. Counting standards were prepared by making 1:50 dilutions of aliquots of the injection solution with 0.85% NaCl.

5. FERROKINETIC STUDIES:

Exactly four hours after the intravenous Fe^{59} was injected, the mouse was decapitated and samples of blood and serum were collected for radiocounting, hematocrit and reticulocyte smear. The spleen was removed and prepared for fresh spleen colony counting (Page 29) and weighing. The right femur and liver were next dissected free of adherent tissue, and placed, as was the weighed spleen, in individual tubes which contained 1 ml. of 10% buffered neutral formalin for radiocounting and fixation. The thymus in control animals, or a block of superior mediastinal tissue from thymectomized animals was fixed in formalin but not radiocounted.

Radioactivity levels were determined in an NaI well-type scintillation counter[1].

6. HEMATOCRIT DETERMINATION:

Hematocrit levels were determined as follows: 75 mm. heparinized capillary tubes having an inside diameter of 1.1 to 1.2 mm. were filled with the mixed venous and arterial blood obtained by decapitation. They were capped and centrifuged for four minutes in a microcapillary centrifuge[2], and read on a circular model reader.

7. CALCULATIONS:

All radioiron uptakes were calculated as percentages of the injected radioactivity. Formulae applied in computing the serum and erythrocyte radioactivity were:

i. Correction of hematocrit (Hct) for trapped plasma = observed Hct x 0.95.

ii. Counts/minute (CPM)/mm^3 whole blood = $\frac{CPM/mgm}{1.056}$

iii. CPM/mm^3 serum = $\frac{CPM/mgm}{1.026}$

1.056 and 1.026 are the specific gravities of blood and serum respectively.

iv. Blood volume in mm^3 = body weight in mgm. x 6.95/100

6.95 is the blood volume expressed as a percentage of body weight (96).

v. RBC CPM/mm^3 whole blood = CPM/mm^3 whole blood - (1-corrected Hct) x CPM/mm^3 serum.

vi. Total RBC CPM = RBC CPM/mm^3 whole blood x blood volume in mm^3.

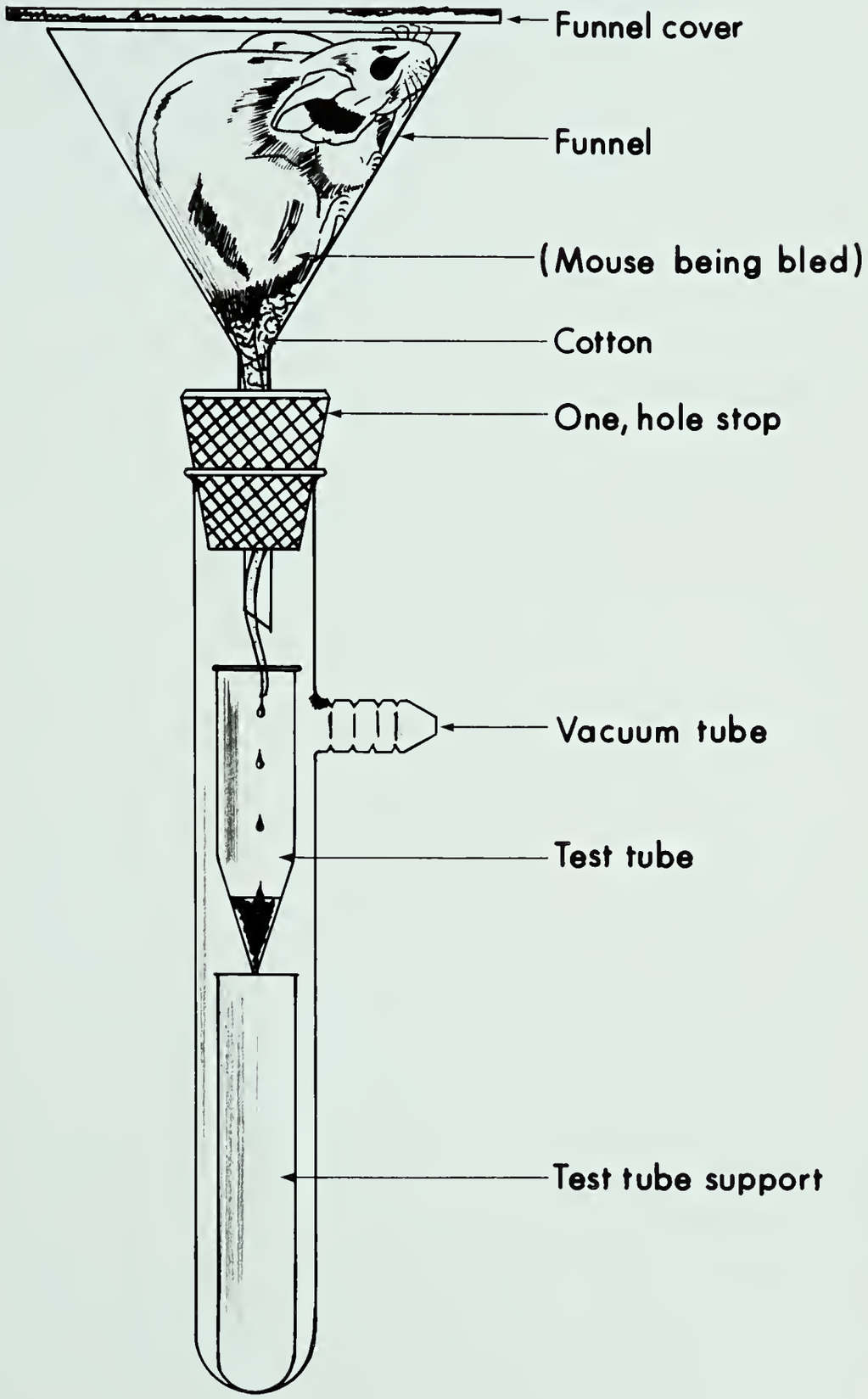
vii. Serum volume in mm^3 = blood volume in mm^3 x (1-corrected Hct).

viii. Total serum CPM = serum volume in mm^3 x CPM/mm^3 serum.

8. BLEEDING:

In order to conduct the experiment which involved the collection of blood, it was deemed important to satisfy the following requirements: firstly, the method should not injure or disable the mouse; secondly, all mice must lose the same volume of blood and there should be no possibility for continued or unrecognized blood loss; thirdly, the samples collected should be suitable for either cell counts or for morphology studies;

BLEEDING APPARATUS



fourthly, in order to avoid variables which might occur as a result of time lag, the procedure should be rapid; and lastly, the procedure should be simple.

None of the usual methods, cardiopuncture, retro-orbital bleeding, or the filling of a capillary tube from tail blood met all these requirements.

An apparatus was devised (Fig. 2) which met these requirements adequately. The mouse was subjected to a light ether anaesthetic, and placed tail first into the funnel. At least 1 mm. was cut off the tip of the tail and the funnel inserted into the suction tube, so that the cut end of the tail rested within a graduated heparin coated inner tube. A piece of wire screen held over the funnel prevented the mouse from escaping. 0.2 to 0.4 ml. of blood could be collected rapidly under suction. Contamination of the sample with urine was not a problem since on the occasions when the mouse voided, the urine was swept into the side stream suction. Applying a thin layer of cotton to the root of the tail as the mouse is placed into the funnel soaks up the urine and further insures that there will be no contamination of the blood.

When the measured amount of blood was obtained, and the suction removed, the cut end of the tail was quickly cauterized with the electrodessicator.

9. COUNTING SPLEEN COLONIES:

Where possible, spleen colonies were counted in both the fresh state and on stained sections projected onto a white surface.

Immediately following removal, the spleen was placed in a clean petri dish and covered with not more than two drops of phosphate buffered saline (PBS) to keep it from drying. The dish was placed on the stage of a stereoscopic dissecting microscope[3] and viewed at nine-fold magnification through an ocular micrometer grid by each of two observers. At this magnification colonies appear as pink-white translucent blisters bulging from the capsule (Fig. 3). All colonies larger than 0.8 x 0.8 mm. were counted. Where the colony counts of the two observers differed by more than two colonies, both recounted the spleen and a mean value of the final counts was obtained. Questionable colonies could be identified by gently stretching the spleen with forceps attached to both ends, or by placing delicate pressure from a forcep tip near the area to be examined. Because of manipulation, color changes, and shrinkage, small colonies were more difficult to identify after five to ten minutes.

After counting, the spleen was rolled momentarily in fresh absorbent tissue paper (Kleenex) to remove excess PBS before weighing.

Not less than five days after fixing in formalin, the whole spleens were embedded in paraffin, and subsequently step sectioned at 50 micron intervals. A minimum of two sections for every 50 microns was mounted on glass slides and stained with hematoxylin and eosin. This resulted in five to six double sections for each spleen. All the sections from each spleen were projected[4] onto a white background. This procedure magnified each section 10.5 times.

Colonies were identified because they were darker than the surrounding tissue, often made a bulge in the surface, and although they

Figure 3 Colour Photographs Demonstrating Spleen Colonies

a. Part of a spleen demonstrating fresh spleen exogenous colonies at approximately 10 times magnification. Specimen is not stained but moistened with PBS. There are at least 8 colonies visible around the rim, and the colony at the tip is probably distorted by manipulation.

b. "Projected spleen colonies": Three sections from a normal spleen inverted atop two serial sections from a spleen which contained seven colonies. Hematoxylin and eosin, magnification approximately 4 times. For counting in the projected system these sections were magnified 10.5 times.

Figure 3a "Fresh Spleen Colonies"

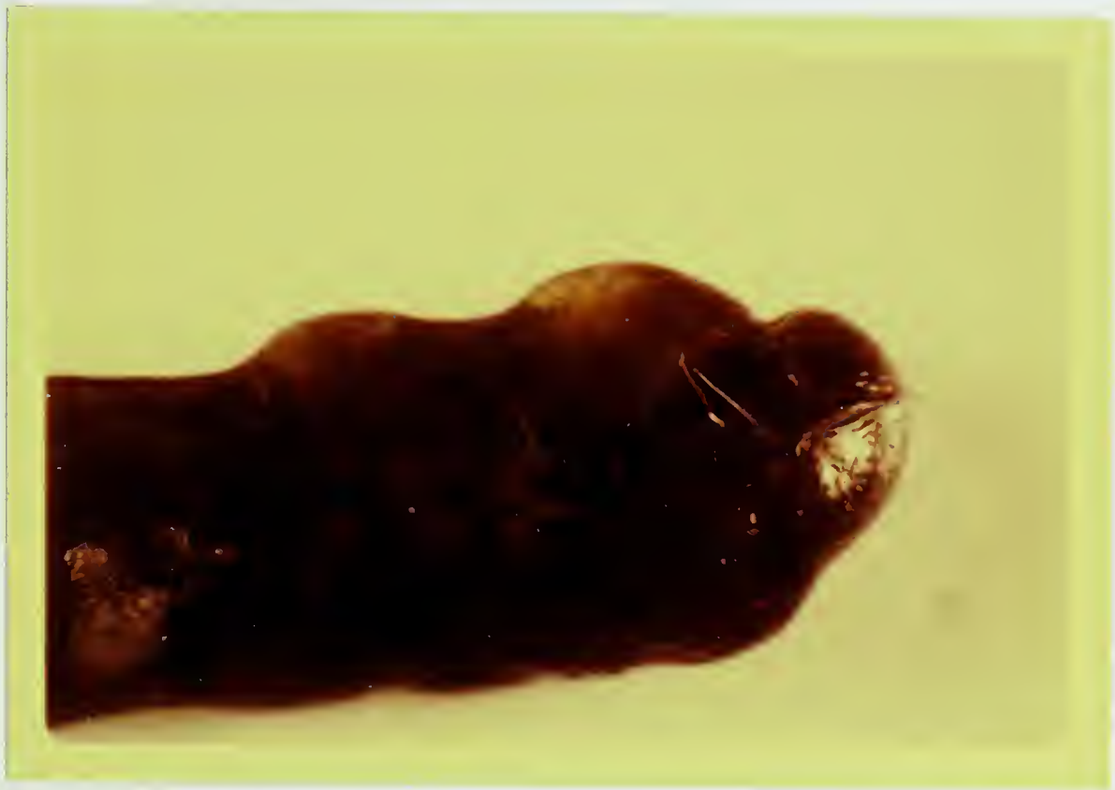


Figure 3b Spleen Sections with Colonies
Compared to Normal Spleen Sections

3c "Projected" spleen colonies: Step sections from one spleen compared by the technique of inverting one slide atop the other. Hematoxylin and eosin, magnification approximately 4 fold. Ten endogenous colonies were counted in this spleen using the projection system which magnified each section 10.5 times.

3d "Projected" spleen colonies: Step sections from one spleen compared by the technique of inverting one slide atop the other. There is less eosin stain present. Hematoxylin and eosin, magnification approximately 4 fold. Nine endogenous colonies were counted in this spleen using the projection system which magnified each section 10.5 times.

Figure 3c "Projected" Spleen Colonies - Step Sections

Figure 3d "Projected" Spleen Colonies - Step Sections

occasionally appeared deeply embedded in the pulp, could always be shown in at least one section to be touching the surface. Only colonies which were clearly distinct and present on at least the two serial sections were counted. Colonies as small as 0.19 x 0.19 mm. could be counted with accuracy by this method. All spleens were counted by the same observer on at least two different occasions, and in every instance where there was a significant difference between test and control groups, by another observer as well.

The routine for counting is as follows: The number of colonies on each spleen section alone was counted, then all the sections from one spleen were projected side by side. Where two discrete colonies were shown in a deeper section to be part of one large colony they were counted as one. As a check, they were finally projected and counted in groups of two, one slide inverted atop the other. In this way, most colonies could be followed through a number of sections, (Fig. 3, b, c, d).

Colonies smaller than 0.04 mm^2 can not be counted by either the fresh or projected section techniques. In order to evaluate the numbers of microscopic (micro.) colonies, the following system was devised.

A microcolony at 100 power magnification, using an ocular micrometer, was defined as one having an area of 31,250 μ^2 (0.03 mm^2) or smaller. The least distance between successive sections is 50 μ (0.05 mm.), and the diameter of a colony of this maximum area is 199 μ (0.2 mm.). Some microcolonies were therefore undoubtedly counted twice.

10. MICROSCOPIC STUDIES:

Fixed sections of spleen, liver, thymus, and femur were routinely examined microscopically to identify any unusual features. No thymic remnants were identified in the superior mediastinal tissue blocks taken from the thymectomized mice, except in one experiment in which thymectomy had been performed by scissor dissection, and five of thirty-four mice had at least part of one lobe intact.

11. BONE MARROW TRANSPLANTATION:

The donor mice were anaesthetized with ether, decapitated, and bled. If the marrow was taken from a thymectomized mouse, the absence of the thymus was verified at this time. Employing aseptic techniques, both femurs and tibia were removed, and the adherent tissue stripped off. Both epiphyses of the bone were cut off, and 1 ml. of phosphate buffered saline was injected via a 22 gauge needle into the marrow cavity. The cells were flushed into a sterile petri dish held in an ice-water bath, and when the required number of marrows had been pooled, the cells were gently suspended by repeatedly drawing them into, and extruding them out of a sterile pasteur pipette.

Large particles and debris were allowed to settle out for five minutes, then the supernatant transferred to a centrifuge tube and subjected to 275 g. for ten minutes. A maximum of 6 ml. of cells and PBS was placed in one centrifuge tube, the cells were washed at 275 g., the supernatant removed, and the pellet washed by resuspending in 6 ml. PBS and recentrifuged as above. The mouths of the centrifuge tubes were plugged with cotton

wool. The final pellet of cells was diluted to 10 ml., resuspended, and the cells counted in a hemacytometer chamber with acetic acid dilutent. The test tube which contained the cells was kept in an ice-water bath during injection. Depending upon the cell yield, the volume injected varied from 0.20 to 0.35 ml. on different days or in different experiments. Viability studies, based on the ability of cells to exclude eosin-Y, were conducted after the injections. Viability was consistently over 94 percent.

12. PREPARATION OF SHEEP RED BLOOD CELLS (SRBC):

In the study using SRBC, 20 ml. of blood was withdrawn from one sheep into two sterile tubes containing acid citrate dextrose anti-coagulant. This blood was kept refrigerated at 4°C and used in 2 ml. amounts. The cells were washed three times prior to use by suspending the blood in 11 ml. PBS and centrifuging for five minutes at 450 g. The pellet was resuspended in 10 ml. PBS and centrifuged as above. Sterile technique was used throughout.

13. SUSPENSION OF ORGAN CELLS:

Spleen and thymus cell suspensions were made by mincing the organ with fine scissors and extruding the cells through a fine mesh, stainless steel grid, into 4 ml. of Eisens Balanced Salt Solution (EBSS). Clumps were further separated by repeatedly drawing the cells into, and expelling them from a sterile pasteur pipette. The cell suspension was allowed to settle for five minutes, and the supernatant was removed and washed three

times in 5 ml. of EBSS.

14. ANTIBODY FORMING CELL ASSAY:

The modified Cunningham microplaque technique was used to estimate the numbers of antibody-forming cells in an antigen stimulated spleen (66). A monolayer of washed spleen cells in suspension with antigen (SRBC) and complement, contained between two glass slides, is incubated for thirty minutes. Clear areas (plaques) appear in the suspension where antibody has hemolysed the surrounding red cells. Freeze dried complement reconstituted to a 1:2 dilution was used, and fresh solution was made each day of the procedure. Approximately 2 - 6 x 10^6 nucleated spleen cells were dispensed per slide, and the numbers of plaques expressed per 10^6 cells. Plaques were counted immediately after incubation through a stereoscopic dissecting microscope[3] at 9 magnifications. The rest of the spleen cells, including the settled debris was counted for its level of radioactivity.

FOOTNOTES

[1] Nuclear Chicago, 2½ inch. NaI (Tl) activated crystal, with pulse height analyser

[2] International microcapillary centrifuge, Model MB

[3] Wild M5 stereo-microscope, Heerbrung, Switzerland

[4] Bausch and Lamb optical projector, Rochester, New York, U.S.A.

IV. EVALUATION OF TECHNIQUES USED TO COUNT SPLEEN COLONIES

1. INTRODUCTION:

Wherever possible, spleen colonies were counted by projecting the images of the spleen sections onto a white background, as was described. In some of the experiments it was necessary to make fresh spleen cell suspensions, and the colonies in these spleens were counted under a dissecting microscope in the fresh state only.

The fresh spleen counts were limited by time, both because mice were killed for study usually at exactly ten minute intervals, and therefore ten minutes was the maximum allowable time for splenectomy and colony counting by each of two observers, and because small colonies were more difficult to identify after five to ten minutes, due to change caused by manipulation, drying, or shrinkage. Forceps or scissor marks could be mistaken for colonies under the dissecting microscope, and furthermore, it became increasingly more difficult to differentiate individual colonies as the numbers increased over fifteen per spleen.

The projection method was not limited by time, and allowed for easy differentiation of colonies from artifact, and colony from colony as confluence in numbers was approached. This method, furthermore, counted colonies too small to be included in the fresh count, and was therefore considered to be the more accurate measurement.

In sixty-five spleens both fresh and projected, colonies were counted. Comparing the number of colonies found by the projection method

with the fresh count method for those spleens which had both types of count, there was no significant difference between the means ($p > 0.30$). When the counts for the sixty-five spleens were compared as pairs using the paired t test, there was again no significant difference ($p > 0.25$). The projected count was highest in 52 percent, the fresh count in 29 percent, but they were equal in only 19 percent of the counts.

Despite the fact that the mean values for the two counting techniques do not differ significantly, it is probable that the fresh counting method undercounts 52 percent of the time, and overcounts 29 percent of the time. The fresh colony count results are therefore not included in the experimental results, except where it was the only counting technique used. These results are included in tables in the appendix.

2. THE RELATIONSHIP BETWEEN SPLENIC Fe^{59} UPTAKE AND SPLEEN COLONY COUNTS:

Schooley (67) reported a good correlation between twenty-four hour spleen radioiron uptake and the numbers of spleen colonies in the exogenous system, and Marsh et al. (68) confirmed that the same is true for endogenous colonies over a wide range of radiation doses. This is because the large surface colonies, i.e. those visible grossly, do not develop in hypertransfused or erythropoietin deprived mice, and are therefore erythropoietic (69).

In the following studies spleen colonies were plotted against percent splenic Fe^{59} uptake, and the curve drawn by inspection. There was a linear correlation between the numbers of colonies and Fe^{59} uptake in every instance regardless of the experimental conditions or whether

colonies were exogenous or endogenous, or counted by the fresh or projected techniques. The line appeared to intersect the Y axis between four to five colonies in all instances, implying that at least this number of non-erythropoietic colonies were included in the colony counts.

Where the correlation between percent spleen Fe^{59} uptake and numbers of spleen colonies is pertinent to the results, then the graph is included and the line fitted mathematically.

3. THE RELATIONSHIP BETWEEN SPLEEN WEIGHT AND SPLEEN COLONIES:

Popp et al. (70), and Smith (71) studied the correlation between spleen weight, iron uptake, and spleen colonies, and found that weight did not correlate well with the other measures of nodule counting. Indeed, Brecher (72) found insignificant spleen weight changes at 400 r, although a colchicine pre-treated group had twice as many spleen colonies.

Marsh et al. (68) had similar results except at 550 r. At this radiation dose the weight correlated well with the number of endogenous colonies observed after ten days. They found that spleen weight was stable to 200 r and then decreased exponentially to 700 r and did not further decrease. They suggest that the initial weight loss represented loss of cells plus a proliferative response to irradiation, and the subsequent plateau reflected the irreducible minimum weight due to radioresistant stroma.

Graphs plotting spleen weight against spleen colonies were made for each of the following studies, and the curves were drawn by

inspection. There appeared to be a linear correlation between spleen weight and numbers of colonies counted even at low (350 r) and high (875 r) doses of total body radiation. This correlation was not influenced by either the method of counting or the experimental circumstances. The spleen weight is included as part of the results in every experiment, but graphs comparing spleen weight and spleen colonies are omitted because they are of limited relevance.

Curry and Trenton (60) step sectioned spleens at 50 μ intervals and counted the microscopic colonies, which they determined were non-erythropoietic. They concluded that the gross counting method is inaccurate, since it overlooks these small colonies. In the studies which follow, spleens were also sectioned at 50 μ, but it has already been noted that some microscopic colonies were undoubtedly counted more than once. Furthermore, there is no statistical proof that the number of microscopic colonies on five or six step sections of a spleen bears a direct relationship to the total number in that spleen. McCulloch (73) feels, not only that counting of microscopic colonies adds nothing to the study of CFU, but also that it defeats the purpose of the assay by making it complicated and time consuming. Microscopic colonies were counted in the hope that they might contribute information to this study, but are only included in the data in instances where fewer than expected numbers of visible colonies were found. No conclusions will be drawn from the numbers of spleen microscopic colonies.

When graphed, there was no correlation between the numbers of microscopic and the numbers of grossly counted colonies, radioiron uptake

or spleen weight. The frequency distribution of the numbers of microscopic colonies conforms to a normal distribution.

V. THE EFFECT OF THYMECTOMY ON ERYTHROPOIESIS AND ENDOGENOUS SPLEEN COLONY FORMATION IN ADULT MICE

Globerson and Feldman (13) reported increased numbers of erythropoietic spleen nodules in thymectomized as compared to normal adult mice, ten days after 550 r total body irradiation. The central purpose of their study was tumor rejection, and their report does not clarify whether the mice used in the spleen colony study were of the same age, sex, strain, or whether or not they had received tumor grafts or other treatment. Both the age of the mouse (74), as well as the introduction of a number of foreign materials (75) are known to influence the numbers of spleen colonies.

The following series of experiments was designed to test the experimental hypothesis that the thymus influences post radiation erythropoietic recovery in an adult mouse, and that this influence is reflected in the numbers of spleen colonies.

Male C3H mice, which are less likely than some other strains to develop post thymectomy autoimmune hemolytic anaemia, were used. Erythropoiesis was measured by femoral bone marrow, red blood cell four hour Fe^{59} uptake, serum Fe^{59} content, and hematocrit and reticulocyte count. Spleen colonies were enumerated by the projection method, spleen Fe^{59} uptake, and spleen weight. It was expected that the radioiron studies would both identify any shifts to extramedullary hemopoiesis, and confirm the erythropoietic nature of the spleen colonies. It was further expected that the best results would be obtained if colonies were counted at a time when there were maximum numbers but before they

became confluent. Consequently, all measurements were performed in any one study on either the eleventh or the twelfth day post irradiation. In order to evaluate the dose dependence of the thymic effect upon erythropoiesis and numbers of spleen colonies, experimental groups were studied following lethal (875 r) and sublethal (600 r, 550 r, 450 r, 350 r) total body irradiation.

Except as otherwise noted, mice used in each study were all of the same age. Where mice of more than one age were included in one study, they were distributed equally into experimental and control groups according to a system of random numbers.

Tables 1, 2, and 3 express the results obtained from groups of thymectomized and sham thymectomized (control) mice eleven days after 350 r, 450 r, and 550 r respectively.

Both experimental and control groups were composed of equal numbers of 56 day old and 72 day old mice. Hematocrit, reticulocyte counts, and the Fe^{59} uptake measurements were obtained from only five mice from each group.

Table 4 expresses the results of an additional experiment eleven days after 550 r but performed three months after the study described in Table 3. All mice were 55 days old, and all had hematocrit, reticulocyte count, and the Fe^{59} studies.

The results obtained when thymectomized and sham thymectomized mice are compared twelve days after 660 r, are expressed in Table 5. All mice were 66 days old and were fully studied as in Table 4.

Table 6 expresses the results obtained twelve days after 875 r.

TABLE 1: Erythropoietic Recovery and Endogenous Spleen Colonies in Thymectomized and Sham Thymectomized Mice After 350 r

GROUP		N	MEAN	S.D.	P
Thym.	Hematocrit	5	36.8	1.94	
Control		5	36.4	1.02	
Thym.	% Reticulocyte	5	1.3	0.06	
Control	Count	5	1.3	0.09	
Thym.	Serum Fe^{59}	5	8.42	5.57	
Control	Content	5	9.30	1.78	
Thym.	RBC Fe^{59}	5	13.22	4.83	
Control	Uptake	5	12.88	3.54	
Thym.	Projected Spleen	14	7.7	2.46	
Control	Colonies	15	7.9	2.79	
Thym.	Spleen	14	61.2	9.59	
Control	Weight	15	58.6	9.05	
Thym.	Spleen Fe^{59}	5	6.3	3.41	
Control	Uptake	5	5.6	0.99	
Thym.	Femur Fe^{59}	5	1.36	0.29	
Control	Uptake	5	1.48	0.20	

TABLE 2: Erythropoietic Recovery and Endogenous Spleen Colonies in Thymectomized and Sham Thymectomized Mice After 450 r

GROUP		N	MEAN	S.D.	P
Thym.	Hematocrit	5	32.8	0.75	
Control		5	31.6	4.22	
Thym.	% Reticulocyte	5	1.08	0.07	
Control	Count	5	1.22	0.08	
Thym.	Serum Fe^{59}	5	16.54	4.70	
Control	Content	5	17.10	6.52	
Thym.	RBC Fe^{59}	5	7.90	3.00	
Control	Uptake	5	6.04	4.16	
Thym.	Projected Spleen	14	6.14	1.96	
Control	Colonies	15	6.53	2.53	
Thym.	Spleen	14	44.44	6.45	
Control	Weight	15	43.21	8.74	
Thym.	Spleen Fe^{59}	5	4.28	1.39	
Control	Uptake	5	3.76	2.04	
Thym.	Femur Fe^{59}	5	1.14	0.44	
Control	Uptake	5	1.24	0.35	

TABLE 3: Erythropoietic Recovery and Endogenous Spleen Colonies in Thymectomized and Sham Thymectomized Mice After 550 r

GROUP		N	MEAN	S.D.	P
Thym.	Hematocrit	4	26.8	2.16	
Control		5	24.2	2.48	
Thym.	% Reticulocyte	5	1.05	0.09	
Control	Count	5	0.99	0.09	
Thym.	Serum Fe^{59}	5	20.50	2.73	
Control	Content	4	24.95	2.40	
Thym.	RBC Fe^{59}	5	1.66	1.15	
Control	Uptake	5	1.26	2.23	
Thym.	Projected Spleen	14	3.71	1.58	
Control	Colonies	13	6.38	2.65	< 0.01
Thym.	Spleen	14	39.04	6.40	
Control	Weight	14	39.27	6.81	
Thym.	Spleen Fe^{59}	5	3.12	1.29	
Control	Uptake	4	2.58	1.23	
Thym.	Femur Fe^{59}	5	0.91	0.26	
Control	Uptake	5	0.69	0.57	

TABLE 4: Erythropoietic Recovery and Endogenous Spleen Colonies in Thymectomized and Sham Thymectomized Mice After 550 r

GROUP		N	MEAN	S.D.	P
Thym.	Hematocrit	10	33.1	2.67	
Control		10	35.6	2.82	
Thym.	% Reticulocyte	10	1.31	0.56	
Control	Count	10	1.45	0.52	
Thym.	Serum Fe^{59}	10	13.84	3.34	
Control	Content	10	15.85	8.86	
Thym.	RBC Fe^{59}	10	4.85	6.23	
Control	Uptake	9	5.47	12.04	
Thym.	Projected Spleen	10	7.60	2.10	<0.025
Control	Colonies	10	10.70	3.16	
Thym.	Fresh Spleen	10	7.80	4.56	
Control	Colonies	10	9.10	3.88	
Thym.	Spleen Micro	10	29.50	7.14	
Control	Colonies	10	30.5	9.80	
Thym.	Spleen	10	39.71	6.19	
Control	Weight	10	45.16	6.02	
Thym.	Spleen Fe^{59}	10	3.84	3.73	
Control	Uptake	10	5.55	3.08	
Thym.	Femur Fe^{59}	10	1.16	0.85	
Control	Uptake	10	1.36	0.49	

TABLE 5: Erythropoietic Recovery and Endogenous Spleen Colonies in Thymectomized and Sham Thymectomized Mice After 600 r

GROUP		N	MEAN	S.D.	P
Thym.	Hematocrit	17	29.18	3.45	
Control		16	30.12	2.45	
Thym.	% Reticulocyte	18	1.61	0.59	
Control	Count	15	1.87	0.66	
Thym.	Serum Fe^{59}	16	18.80	6.12	
Control	Content	16	16.58	4.18	
Thym.	RBC Fe^{59}	16	6.98	6.33	
Control	Uptake	16	8.64	4.29	
Thym.	Projected Spleen	17	6.65	3.16	
Control	Colonies	16	8.19	2.70	
Thym.	Spleen	17	34.05	7.89	
Control	Weight	16	36.15	5.49	
Thym.	Spleen Fe^{59}	17	6.65	3.16	
Control	Uptake	16	8.19	2.70	
Thym.	Femur Fe^{59}	17	1.37	0.41	
Control	Uptake	16	1.39	0.35	

TABLE 6: Erythropoietic Recovery and Endogenous Spleen Colonies in Thymectomized and Sham Thymectomized Mice After 875 r

GROUP		N	MEAN	S.D.	P	ADJUSTED MEAN	ADJUSTED S.D.	ADJUSTED P
Thym.	Hct.	4	15.8	4.87		3.92	0.61	
Control		6	13.3	2.36		3.64	0.32	
Thym.	% Retic.	5	6.9	4.69		2.41	1.05	
Control		6	3.6	0.90		1.87	0.24	
Thym.	Serum Fe^{59}	4	7.89	3.07	0.001	2.76	0.51	<0.001
Control		6	30.60	8.64		5.47	0.83	
Thym.	RBC Fe^{59}	4	- 0.32	0.45	0.01	-0.44	0.35	<0.01
Control		6	- 2.28	1.11		-1.44	0.46	
Thym.	Projected Spleen	5	1.6	0.49		1.25	0.20	
Control	Colonies	6	2.0	1.41		1.24	0.67	
Thym.	Spleen	5	29.28	4.73		5.39	0.44	
Control	Weight	6	24.50	3.14		4.94	0.33	
Thym.	Spleen Fe^{59}	4	0.06	0.04		0.23	0.08	<0.02
Control		6	0.50	0.47		0.64	0.29	
Thym.	Femur Fe^{59}	4	0.06	0.03		0.22	0.07	
Control		6	0.20	0.17		0.40	0.19	

All mice were 54 days old. Eight of the thymectomized (62 percent), and one control mouse (14 percent) died prior to the study time. Surviving mice demonstrated marked loss of subcutaneous fatty tissue, were lethargic, had hemorrhagic gastritis, bowel petechiae, and occasional muscular and subcutaneous hematomata. The body weight of survivors was the same for both groups, but had diminished 13 percent from pre-irradiation levels. The spleen colonies on the twelfth day were very small. In addition, technical problems were encountered in the reticulocyte smears and stain, and reticulocyte counts are therefore not included in Table 6.

The results obtained from studying groups of mice at 350 r, 450 r, 550 r, and 600 r levels of total body radiation (Tables 1 - 5) may be summarized as follows. Comparing thymectomized to sham thymectomized mice, there is no difference in erythropoiesis as measured by hematocrit, reticulocyte count, or Fe^{59} uptake in the circulating red blood cells, the spleen, or the femur at any of these irradiation doses. At 550 r only, there are significantly fewer spleen colonies in the thymectomized mice (Tables 4 and 5).

Since Graph 1 shows a linear relationship between projected spleen colonies and percent spleen uptake of Fe^{59}, it is reasonable to assume that increased numbers of spleen colonies would result in increased Fe^{59} uptake. That this is not the case cannot therefore be attributed entirely to a lack of sensitivity in the Fe^{59} assay, and it is probable that the decreased numbers of spleen colonies in the thymectomized mice at 550 r represents non-erythropoietic colonies.

Evidence has been presented to demonstrate that the numbers

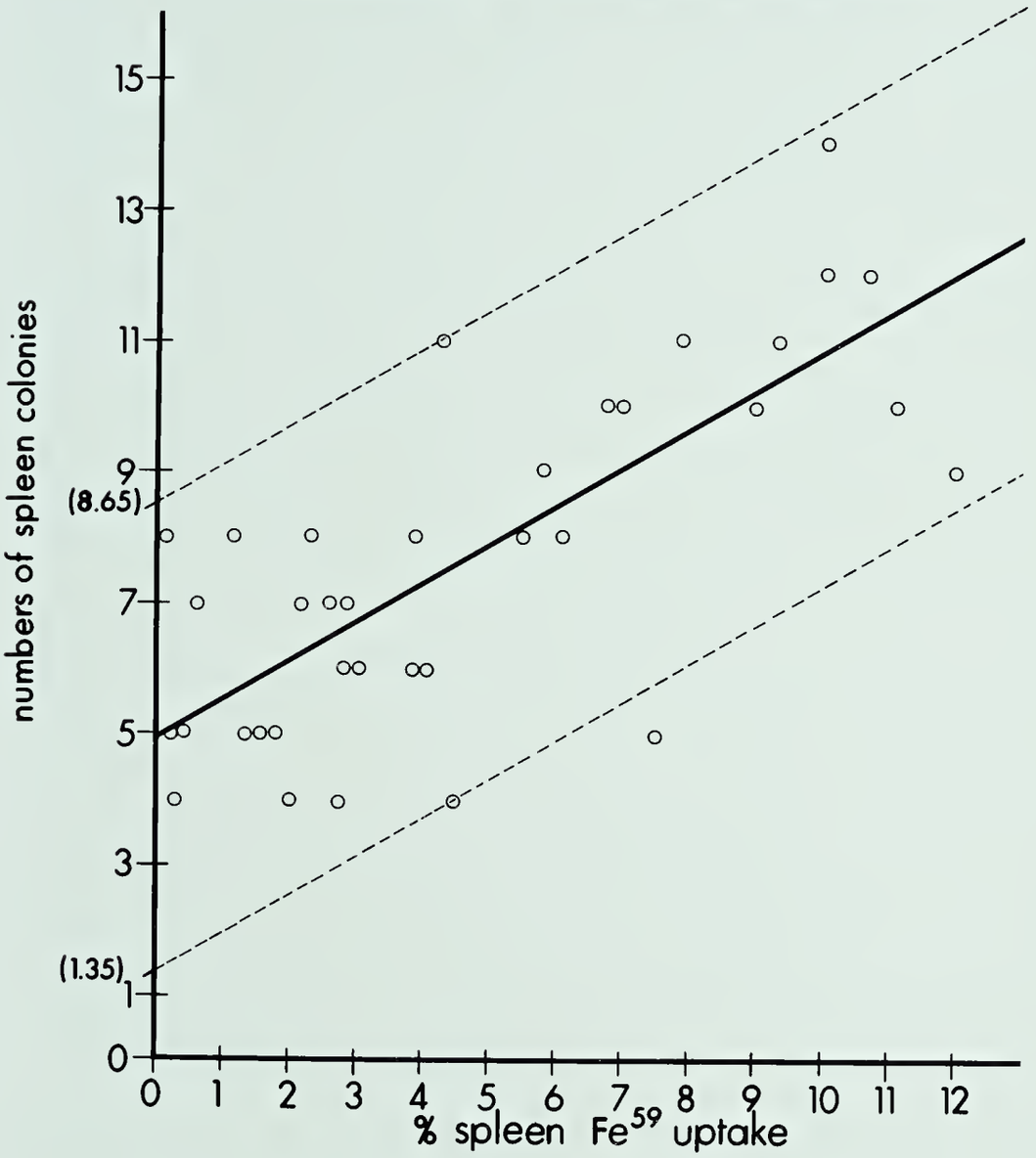
Figure 4: The number of spleen colonies as counted on projected step sections (Y axis) compared to the percentage uptake of radioactive iron in the spleens four hours after intravenous injection of Fe^{59} complexed with normal mouse serum (X axis).

Each point on the graph represents the values determined from one spleen. All animals were C3H male mice 66 $\pm$ 3 days old on the day of irradiation. Animals were subjected to 550 r in three groups of 12 mice each, the last group being irradiated within 30 minutes of the first group.

In the curve of best fit as determined by the method of least squares, the Y intercept is at 5 spleen colonies. The 95% confidence limits for the numbers of colonies are included.

FIGURE 4

The correlation between numbers of endogenous spleen colonies and spleen % Fe^{59} uptake 11 days after 550 r.



of spleen colonies are directly proportional to the available numbers of CFU. The possibility nevertheless exists that in any one set of experimental conditions there may be fewer spleen colonies counted, despite unchanged numbers of CFU in the animal as a whole. This might occur if CFU were either prevented from entering the spleen, or preferentially channeled away from it. It might also occur if CFU were arrested in their development, or if colonies lost their cells, i.e. to the peripheral blood, as they matured. In the latter two cases the colony counts might be low only because the colony size is too small to be seen grossly. The spleen microscopic colony count was therefore performed on the spleens from Table 4 to see if the reduction in numbers of macroscopic colonies might be matched by an increase in microscopic colonies, but the number of estimated microscopic colonies was the same in both groups.

The results obtained at 875 r (Table 6) are not consistent with the previous studies because there was decreased radioiron in the spleens and serum, and increased uptake in the red blood cells of the thymectomized mice. At lower radiation doses the serum radioiron uptake decreased as the splenic uptake increased, implying that serum radioiron was a measure of residual or not-utilized iron. At 875 r the converse is true, i.e. when serum iron is plotted against spleen iron uptake, an increase in one corresponds with an increase in the other. It would be consistent with the increased mortality in the thymectomized mice to speculate that serum radioiron was being lost. Furthermore, while the amount of radioiron recovered from one femur, the spleen, and circulating blood in the other studies varied from 24

to 35 percent at different radiation doses, there was no difference in recovery between the thymectomized and control mice at any one radiation dose. At 875 r however, 29 percent was recovered from the control mice, but only 8 percent from the thymectomized mice. Both groups of mice showed evidence of bleeding on autopsy. If the thymectomized mice were bleeding more than the control mice, yet maintained the same hematocrit, then perhaps the increased red cell radioactivity reflects the loss of splenic red cells, and accounts for the lower splenic radioactivity in the thymectomized mice.

CONCLUSIONS:

It is concluded firstly, that erythropoietic recovery following 350 r, 450 r, 550 r, 650 r, or 875 r total body radiation is not affected by prior thymectomy. Secondly, at 550 r there are significantly fewer endogenous spleen colonies in thymectomized mice, and the evidence suggests that the loss is of non-erythropoietic colonies.

VI. THE EFFECT OF POST IRRADIATION BLEEDING ON ERYTHROPOIETIC RECOVERY AND ENDOGENOUS SPLEEN COLONY FORMATION IN THYMECTOMIZED AND SHAM THYMECTOMIZED MICE

The effects of erythropoietin on spleen colonies and bone marrow colony forming units have been summarized by Curry et al. (69). They demonstrated, as have other investigators (76) (77) (78) (79), that erythropoietin or erythrocyte stimulating factor (ESF) is essential for the growth of both endogenous and exogenous erythropoietic colonies. Macroscopic erythropoietic colonies were eliminated by hyper-transfusion polycythaemia, but underdeveloped erythropoietic colonies of microscopic size could still be identified in the spleens. Treatment of a hyper-transfused mouse with exogenous erythropoietin results in normal macroscopic colony development, and treatment of the irradiated non-plethoric mouse with antiserum to erythropoietin depresses the numbers of macroscopic colonies (77) (80). None of the authors found evidence to suggest that non-erythropoietic colonies were influenced by erythropoietin, or that the numbers of these colonies were increased if erythropoietin was suppressed and erythropoietic colonies unable to develop.

It must be assumed from this evidence that erythropoietin does not act upon the CFU but on a population of erythropoietin sensitive cells some generations removed from it. There is no increase in available CFU after erythropoietin stimulation, and Schooley et al. (78) have produced evidence which shows that repopulation of the erythropoietin sensitive cells has priority over restoration of CFU. O'Grady et al. (81) compared cell renewal times with the rate of growth of erythropoietic colonies in plethoric mice, and concluded that erythropoietin might act on

a population of cells eight to ten generations removed from the original stem cell.

Accumulating evidence (48) (79) (81) (82) (83), indicates that the erythropoietin sensitive cell and the CFU are two different cells. Erythropoietin acts upon a cell committed to the erythron, not multipotential but still immature. In the absence of erythropoietin, CFU can undergo eight to nine doublings into cells committed to erythropoiesis.

Curry et al. (84) gave exogenous erythropoietin to mice which had been subjected to 580 r total body irradiation, and since this did not increase the proportion of erythroid colonies, they concluded that the erythropoietin levels were maximal.

In the experiments reported in this thesis (Tables 3 and 4), it was assumed that the erythropoietin stimulation which resulted from radiation induced anaemia, was equal, and at 550 r maximal in both the thymectomized and control animals.

The control animals in those experiments had more endogenous spleen colonies than thymectomized animals, after 550 r. It was therefore important to test the assumption that erythropoietin levels were equal and maximal at this dose.

Marsh et al. (85) have shown that bleeding is a more effective stimulus for spleen colony formation than erythropoietin, testosterone, cobalt, or a cobalt-testosterone combination. Therefore, the following experiment was designed to examine the effect of post-irradiation bleeding on erythropoietic recovery and endogenous spleen colonies after 550 r.

It was expected that if erythropoietin levels were maximal,

that the hematocrit would be significantly lowered, and that the spleen colonies would not be increased in the bled animals.

A group of thymectomized and sham thymectomized (control) 66 - day old C3H male mice was subjected to 550 r. Half of the thymectomized and half of the control mice were bled 0.3 ml. on both the fourth and the eighth day post irradiation. Femur, spleen, and red blood cell Fe^{59} uptakes, serum radioiron, hematocrit and reticulocyte counts, and spleen colony counts were done eleven days post irradiation. The results comparing bled and not-bled control mice are listed in Table 7. Bled and not-bled thymectomized mice are compared in Table 8. Bled thymectomized are compared to bled control mice in Table 9.

The results may be summarized as follows: There was no difference in femur, spleen, or red blood cell erythropoiesis as measured by Fe^{59} uptake, between bled and not-bled mice, and the hematocrits of the bled group were in both cases (Table 7 and 8) significantly lower than the not-bled group. Tables 7, 8, and 9 demonstrate that this effect is independent of the thymus.

There was a higher percentage of reticulocytes in the peripheral blood of the bled mice compared to the not-bled mice. Absolute reticulocyte counts were not performed, however it is obvious the animals with the lowest hematocrits have the highest percentage of reticulocytes. When the percentage of reticulocytes was multiplied by the hematocrit obtained from the same mouse, there was no difference between the experimental groups. It is concluded from this that the difference in percentage of reticulocytes probably does not reflect

TABLE 7: Post Irradiation Erythropoietic Recovery and Spleen Colony Formation in Bled Versus Not-Bled Control Mice

GROUP		N	MEAN	S.D.	P
Bled	Hematocrit	7	19.1	3.74	<0.001
Not Bled		10	35.6	2.82	
Bled	% Reticulocyte	7	2.84	1.34	<0.05
Not Bled	Count	10	1.45	0.52	
Bled	Serum Fe^{59}	7	18.02	4.01	
Not Bled	Content	9	15.85	8.86	
Bled	RBC Fe^{59}	7	9.91	4.80	
Not Bled	Uptake	9	5.47	12.04	
Bled	Projected Spleen	7	6.6	1.84	<0.01
Not Bled	Colonies	10	10.7	3.16	
Bled	Fresh Spleen	7	9.14	2.90	
Not Bled	Colonies	10	9.10	3.88	
Bled	Spleen	6	50.45	7.54	
Not Bled	Weight	10	45.16	6.02	
Bled	Spleen Fe^{59}	6	6.01	2.71	
Not Bled	Uptake	10	5.55	3.08	
Bled	Femur Fe^{59}	7	1.35	0.26	
Not Bled	Uptake	10	1.36	0.49	

TABLE 8: Post Irradiation Erythropoietic Recovery and Spleen Colony Formation in Bled Versus Not-Bled Thymectomized Mice

GROUP		N	MEAN	S.D.	P
Bled	Hematocrit	10	17.28	5.08	<0.0001
Not Bled		10	33.14	2.67	
Bled	% Reticulocyte	10	2.18	1.02	<0.05
Not Bled	Count	10	1.31	0.56	
Bled	Serum Fe^{59}	10	22.70	8.76	<0.02
Not Bled	Content	10	13.84	3.34	
Bled	RBC Fe^{59}	10	7.62	6.99	
Not Bled	Uptake	10	6.22	4.85	
Bled	Projected Spleen	10	6.9	2.77	
Not Bled	Colonies	9	7.1	1.59	
Bled	Fresh Spleen	10	7.9	5.59	
Not Bled	Colonies	10	7.8	4.56	
Bled	Spleen	10	44.89	15.37	
Not Bled	Weight	10	39.71	6.19	
Bled	Spleen Fe^{59}	10	3.96	3.29	
Not Bled	Uptake	10	3.84	3.73	
Bled	Femur Fe^{59}	10	1.15	0.45	
Not Bled	Uptake	10	1.16	0.84	

TABLE 9: Post Irradiation Erythropoietic Recovery and Spleen Colony Formation in Bled Thymectomized Versus Bled Control Mice

GROUP		N	MEAN	S.D.	P
Thym.	Hematocrit	10	17.3	5.08	
Control		7	19.1	3.74	
Thym.	% Reticulocyte	10	2.18	1.02	
Control	Count	7	2.84	1.34	
Thym.	Serum Fe^{59}	10	22.70	8.75	
Control	Content	7	18.02	4.01	
Thym.	RBC Fe^{59}	10	7.62	6.99	
Control	Uptake	7	9.91	4.80	
Thym.	Projected Spleen	10	6.90	2.77	
Control	Colonies	7	6.57	1.84	
Thym.	Fresh Spleen	10	7.9	5.59	
Control	Colonies	7	9.1	2.90	
Thym.	Spleen	10	44.89	15.37	
Control	Weight	6	50.45	7.54	
Thym.	Spleen Fe^{59}	10	3.96	3.29	
Control	Uptake	6	6.01	2.71	
Thym.	Femur Fe^{59}	10	1.15	0.45	
Control	Uptake	7	1.35	0.26	

differences in absolute numbers of reticulocytes or imply differences in erythropoietic stimulation.

Bleeding reduced the numbers of macroscopic spleen colonies in the control but not in the thymectomized mice, and as in the previous set of experiments, the loss appears to be of non-erythropoietic colonies, since there is no change in splenic radioiron uptake. It would seem from the combined results of Tables 3, 4, 7, 8, and 9 that thymectomy has a similar effect on spleen colony formation as post-irradiation bleeding. There are however inconsistencies between the counting methods (projected versus fresh counting methods) such that one may doubt the biological significance of the statistical difference shown.

It might be speculated however, that the reduction in numbers of spleen colonies with bleeding represents a preferential regeneration of erythropoietin sensitive cells at the expense of CFU.

The not-bled thymectomized and not-bled control mice reported earlier (Table 4) were part of this study and are therefore of the same age, sex, and strain as these mice. It is interesting to note therefore that bleeding removed the advantage which the control mice maintained in spleen colony formation (Table 9).

Spleen microcolonies were counted, but because of the difficulty in interpreting them they are not included but are listed in the appendix.

Serum Fe^{59} was also higher in the bled thymectomized compared to the not-bled thymectomized group. Serum iron levels were shown

earlier to vary inversely with splenic radioiron uptake, implying that this represents iron which remains unutilized because of diminished erythropoiesis elsewhere, except in one study (Table 6) where it was postulated that serum was being lost during the four hour iron study. Neither of these explanations reasonably explains the increased serum radioiron levels in the bled thymectomized mice, and there is no reason to assume a contracted circulating plasma volume. The increase in serum radioiron levels in the bled thymectomized mice in this study might therefore represent diminished total body erythropoiesis, but since there is no decrease in femur, spleen, or circulating red blood cell Fe^{59} uptake, this finding must be interpreted with caution.

Marsh et al. (85) irradiated normal adult mice with 700 r and then bled them 0.4 ml. once, at either one or six hours, one, three, or seven days later. They found that ten days after irradiation, bleeding had shown no effect on splenic hematopoiesis as measured by both spleen colonies and spleen Fe^{59} uptake, though there was a significant reduction in the volume of packed red blood cells in the bled compared to not-bled mice. Mice not bled, but treated with large daily doses of erythropoietin after 700 r radiation had significantly more spleen colonies, heavier spleens, and increased radioiron uptake when studied ten days after irradiation.

Superficially, the findings of Marsh et al. can be reconciled with those of other investigators, if, as they suggest, their erythropoietin was more potent. If one further assumes that there is a limit to the amount of erythropoietin which one animal can produce and liberate

but that at this maximum there still exist pools of unstimulated erythropoietin sensitive cells, then further bleeding would not result in increased erythropoiesis but exogenous erythropoietin would.

A number of investigators (85) (86) (87) have suggested that the bleeding stimulus is different from erythropoietin. Generally, the reasoning follows that bleeding stimulates all the elements of the hemopoietic system, and very rapidly, but that erythropoietin stimulates only the erythropoietic sensitive cells and is not present at discernable levels until several hours after bleeding.

CONCLUSIONS:

Bleeding a mouse which was subjected to 550 r total body radiation significantly lowered the hematocrit and did not alter either the femur, spleen, or red blood cell Fe^{59} uptake compared to not-bled controls. Spleen colonies were furthermore not increased in numbers, and the absolute reticulocyte counts were probably not increased by bleeding. It is concluded therefore, that bleeding did not further stimulate the post irradiation erythropoiesis, and that the stimulus to erythropoiesis is therefore already maximal.

VII. POST RADIATION ERYTHROPOIETIC RECOVERY AND SPLEEN COLONY FORMATION IN BONE MARROW REPLACED, THYMECTOMIZED AND SHAM THYMECTOMIZED MICE

The mechanisms through which the thymus influences the immune response are not fully understood. If the response is dependent upon an endocrine function of the thymus, or upon cellular or antigenic contact with viable thymus cells, then thymectomy would ablate immune responsiveness. This indeed is the result of neonatal or embryonic thymectomy, but adult thymectomy does not eliminate immune responsiveness. The probable reason for this, as stated earlier, is that immune responsiveness resides in the long lived circulating lymphocytes which maintain immune competence by virtue of prior contact with the thymus, and in the preformed ARC in which there is immunologic memory. Total body radiation of a thymectomized adult animal makes it an immunological cripple similar to the neonatally thymectomized animal, presumably by injuring the preformed thymus dependent cells.

The degree to which the immune response is impaired depends upon the dose of radiation, and Globerson and Feldman (13) found that more than 50 percent of C3H mice which had been thymectomized and subjected to 550 r were able to reject a tumor homograft which had been grafted forty days after radiation. Despite the fact, therefore, that following thymectomy there is a gradual but persistent loss of lymphocytes from the spleen, lymph nodes, thoracic duct, and circulating blood (2), it may be assumed that some AFC are present in the spleens of thymectomized radiated mice. It is possible that the thymus exerts an effect upon erythropoiesis through the mediation of the persisting

"thymus-dependent" cells, and that adequate numbers of these cells are viable eleven days after irradiation to obscure the abnormal increase and persistence of splenic erythroblasts which Miller (40) noted in thymectomized embryonic opossums.

Curry et al. (88), and Wolf et al. (89) concluded that the environment within the spleen affects the type of colony which develops. They performed a complex series of experiments in which they studied the erythroid to granuloid (E:G) colony ratio in the spleens of lethally irradiated bone marrow transplanted mice, studied endogenous and exogenous bone marrow regeneration after heavy radiation, performed colony retransplantation studies and observed the E:G ratios on the spleen colonies which developed in spleens that contained grafts of bone marrow stroma. They postulate that the spleen contains at least four hemopoietic-inductive microenvironments (HIM), and these determine the differentiation of CFU into either erythropoietic, granulopoietic, megakaryopoietic, or eosinophilic colonies. These studies further lead them to conclude that mixed colonies occur when one colony expands into the HIM of an adjacent but different HIM, that the effect of the HIM probably involves cell to cell contact, and that in the case of erythropoietic colonies the erythroid HIM probably renders the CFU sensitive to erythropoietin.

Since the spleen is a mixture of thymus derived and bone marrow derived cells, (4), it is possible that the thymus derived cells could influence the HIM.

If an experimental model could be designed therefore to treat two groups of mice in such a way that the experimental group would be

totally devoid of thymus derived cells but the control group be normally immune competent, then these two groups could be subjected to the same post radiation erythropoietic recovery and spleen colony studies performed as before.

It was reasoned that this model might be achieved if a group of mice were thymectomized and subjected to 550 r so that they would suffer an irreplaceable loss of immune-competent cells. After two months bone marrow from these mice (even though it might be contaminated by circulating blood) would be expected to contain only trace amounts of immune-competent cells. Injection of this bone marrow into thymectomized or normal, lethally radiated isogeneic hosts would produce an animal totally unable to develop new thymic-dependent immune competent cells in the former, but fully capable of developing these cells in the latter case. If 700 r reduces the spleen to its minimum weight, which is due to the radioresistant stroma (68), then 800 r should insure that no thymus derived cells would be still viable in the host mouse.

The following experiment was therefore designed (Fig. 5) to measure endogenous spleen colonies and erythropoietic recovery in mice which would have a much reduced thymus influence.

Donor mice were thymectomized and seven days later subjected to 550 r total body radiation. After a post irradiation recovery period of eight weeks, 5×10^6 femoral and tibial bone marrow cells from these mice was injected intravenously into each of 42 thymectomized and 42 sham thymectomized lethally irradiated mice of the same strain. Five

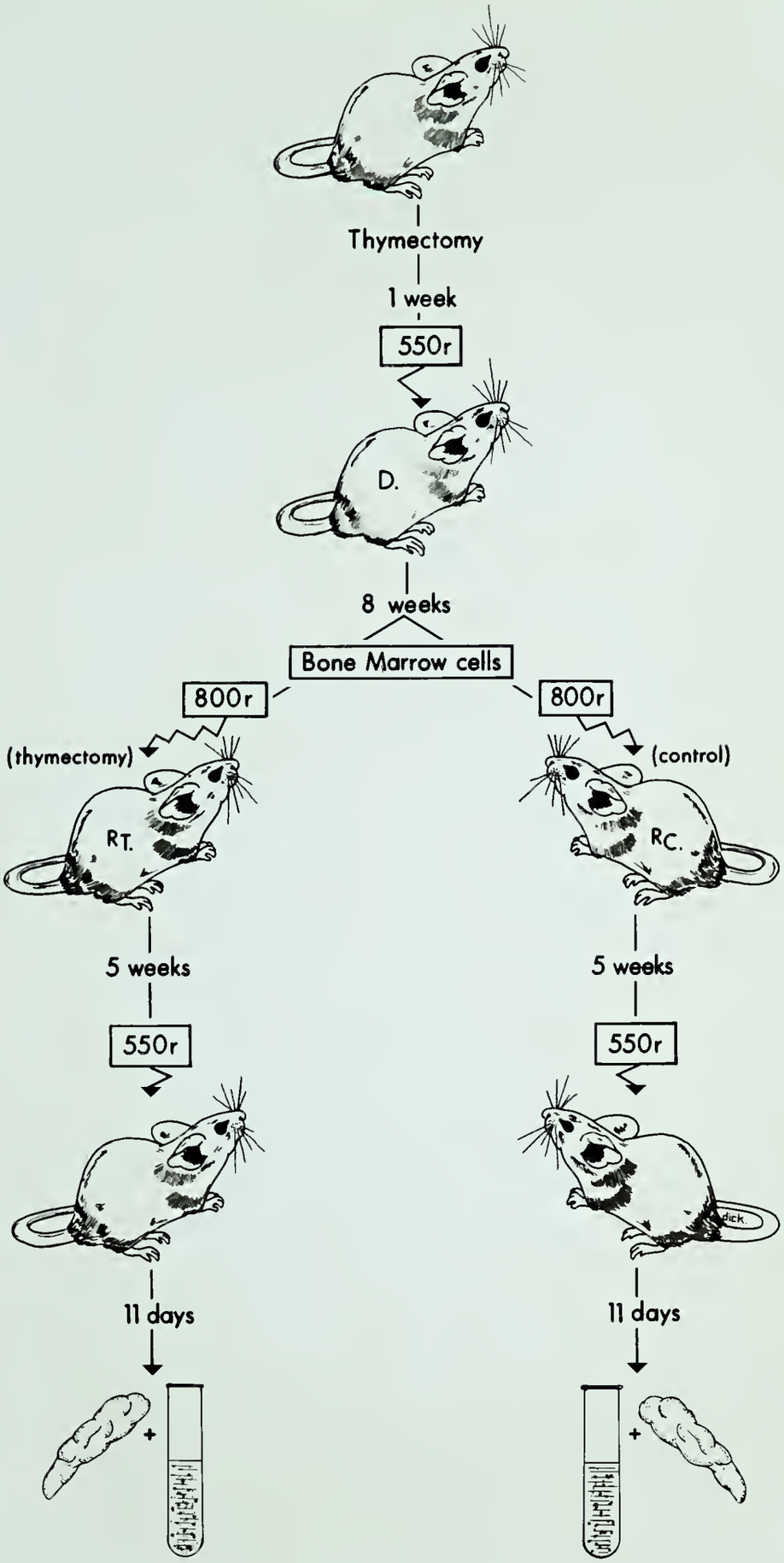
Figure 5: Design of Experiment to Compare Spleen Colony Formation and Erythropoietic Recovery in Mice Thoroughly Devoid of Thymic Influence and Mice with Intact Thymuses

Donor mice are thymectomized and 7 days later subjected to 550 r total body irradiation.

Eight weeks later they are used as donors of 5×10^6 femoral and tibial bone marrow cells for thymectomized and sham thymectomized lethally irradiated mice of the same strain.

Five weeks later all recipient mice are subjected to 550 r total body irradiation.

Eleven days later all recipients are subjected to erythropoietic and endogenous spleen colony studies.


Thymectomy
1 week
550r
D.
8 weeks
Bone Marrow cells
800r
800r
(thymectomy)
(control)
RT.
RC.
5 weeks
5 weeks
550r
550r
11 days
11 days
+
+

weeks after bone marrow grafting each recipient mouse was subjected to 550 r total body radiation. The bone marrow donors were 106 days old at the time of transplantation, the recipient mice were 128, 111, and 78 days old respectively on the second radiation day. These mice were sorted into experimental groups according to a system of random numbers. Endogenous spleen colonies, spleen, femur, and red blood cell Fe^{59} uptake, spleen weight, and serum radioactivity were measured eleven days after the second radiation.

It was technically not possible to lethally irradiate and prepare and graft the bone marrow cells of such a large group of mice all in one day, consequently the mice were separated into three experimental groups, each one treated identically but on consecutive days at each stage of the experiment. For convenience, these are referred to as first group, second group, and third group mice.

There was an overall mortality of 61 percent following lethal irradiation and bone marrow grafting. Most of the deaths occured five to twelve days after bone marrow grafting, and none occurred after the twenty-eighth day. Exogenous colonies are believed to reflect the numbers of CFU in the inoculum and it is expected that all of the CFU which lodge in the spleen do so within a short time after innoculation. The endogenous system, on the other hand reflects total body CFU and it is believed that CFU are constantly seeding the spleen from the bone marrow. Of the first group and second group mice which died in the post irradiation period, it is of interest to note (Table 15 Appendix) that fifty percent had zero, thirty percent had one only, and none had more than three exogenous spleen colonies. Microscopic examination

of the spleen sections revealed that the low numbers of colonies did not reflect confluence, since the splenic red pulp was hypocellular. As predicted, it means that the bone marrow innoculum injected into these animals had very few CFU. In the third group mice which died, there was an average of eleven exogenous spleen colonies. Since radiation survival correlates well with the numbers of spleen colonies, it is of interest to note that of the combined first and second group mice seventy-four percent died but only thirty-five percent of the third group mice died.

There was no difference between the first and second group mice, whether they were compared day by day or as a group, comparing death rates, overall mortality, numbers of spleen colonies, or on post mortum examination. The third group mice however, obviously received more CFU in their bone marrow graft, and the reason for this is not clear. Because of this however, the results of the first and second group mice are combined in Table 10, and the third group mice are included separately in Table 11.

This experiment was designed to compare spleen colony formation and erythropoietic recovery between two groups of mice, one of which had presumably a much reduced thymic influence, and the other which had an intact thymus. The results listed in Tables 10 and 11 show no difference in erythropoiesis as measured by red blood cell, spleen, or femur Fe^{59} uptake, hematocrit or reticulocyte count. There is no difference in spleen colonies or serum radioactivity.

That there were not decreased numbers of non-erythropoietic spleen colonies in this study, despite the fact that it was performed

TABLE 10: Post Radiation Erythropoietic Recovery and Spleen Colony Formation in Bone Marrow Replaced Thymectomized and Sham Thymectomized Mice

GROUP		N	MEAN	S.D.	P
Thym.	Hematocrit	4	22.5	3.57	
Control		8	24.5	3.94	
Thym.	% Reticulocyte	4	0.8	0.04	
Control	Count	8	0.9	0.18	
Thym.	Serum Fe^{59}	4	31.72	9.83	
Control	Content	8	27.54	6.81	
Thym.	RBC Fe^{59}	4	-4.72	2.14	
Control	Uptake	8	-1.66	4.85	
Thym.	Projected Spleen	4	4.75	2.28	
Control	Colonies	8	5.88	3.48	
Thym.	Spleen	5	27.76	4.46	
Control	Weight	9	31.31	8.54	
Thym.	Spleen Fe^{59}	4	1.12	0.16	
Control	Uptake	8	3.08	2.84	
Thym.	Femur Fe^{59}	4	0.53	0.19	
Control	Uptake	8	0.56	0.34	

TABLE 11: Post Radiation Erythropoietic Recovery and Spleen Colony Formation in Bone Marrow Replaced Thymectomized and Sham Thymectomized Mice

GROUP		N	MEAN	S.D.	P
Thym.	Hematocrit	5	25.4	3.14	
Control		12	23.0	6.26	
Thym.	% Reticulocyte	5	1.1	0.12	
Control	Count	12	1.2	0.16	
Thym.	Serum Fe^{59}	5	23.08	4.15	
Control	Content	12	25.78	8.45	
Thym.	RBC Fe^{59}	5	-0.97	2.60	
Control	Uptake	12	-1.29	3.25	
Thym.	Projected Spleen	5	7.60	1.85	
Control	Colonies	12	8.25	3.51	
Thym.	Spleen	6	35.99	12.78	
Control	Weight	12	32.80	5.70	
Thym.	Spleen Fe^{59}	5	2.96	1.51	
Control	Uptake	12	4.08	3.32	
Thym.	Femur Fe^{59}	5	0.77	0.26	
Control	Uptake	12	0.94	0.36	

at 550 r, may be due to the use of mice of mixed ages.

CONCLUSION:

It is concluded that, under the conditions of this experiment, the thymus exerted no influence upon either spleen colony formation or erythropoietic recovery.

VIII. THE EFFECT OF BLEEDING AND SHEEP RED BLOOD CELLS (SRBC) UPON ERYTHROPOIETIC AND IMMUNOLOGIC RECOVERY AND ENDOGENOUS SPLEEN COLONY FORMATION

It is possible to separate cells involved in the immune response into three distinct classes: stem cells, antigen reactive cells (ARC), and antibody forming cells (AFC). It is generally believed that stem cells which do not respond to antigen, differentiate to form cells which are sensitive to stimulation by antigen (ARC). In a current model of the steps involved in immune responsiveness as discussed by Roitt et al. (90), it appears that there are two distinct populations of ARC. The cells of the thymic-dependent (T-lymphocyte) population divide upon contact with antigen and transform into large blast cells which then, through redivision may form long lived memory cells, or produce soluble factors which perform the functions associated with delayed hypersensitivity. It is possible that the T-lymphocyte will only respond to antigen which has been processed by macrophages. Neither the T-lymphocyte or its progeny is believed capable of forming antibody. The thymic-independent cells (bursa or B-lymphocytes) form the other population. These are ARC, which on contact with antigen, differentiate, proliferate, and mature into plasma cells which synthesize humoral antibody (AFC). It has been determined by Kennedy et al. (91), and others, that bone marrow, fetal liver, and thymus do not contain ARC, but that spleen and lymph nodes do; that the progenitors of the ARC exist in the bone marrow, and that their differentiation into at least the T-lymphocyte ARC depends

upon the presence of an intact thymus (94). Abdou and Richter (95) have recently shown that bone marrow cells of normal, unimmunized rabbits can react with blastogenesis and mitosis and tritiated thymidine incorporation upon exposure to a variety of protein antigens in vitro. This implies that ARC do indeed exist in the bone marrow.

The possible relationship between the CFU and the ARC or its precursor, as well as the relationship between the CFU and the erythropoietin sensitive cell was discussed. It was further noted that some of the experimental studies which related the thymus to erythropoiesis might be explained on the basis of immunologic or thymic competition for a stem cell common to both the erythropoietic and lymphopoietic cell lines.

CFU can be assayed by colony formation, and erythropoietin sensitive cells by Fe^{59} uptake, but there is no direct assay for the ARC. Kennedy et al. (91), and Playfair et al. (92), who reasoned that the transition from ARC to AFC involved cellular proliferation (93), expected that the spleens of mice treated with antigen should contain small colonies of AFC which could be detected as a focus of antibody-producing activity. Such foci were found and shown to be linearly related to the number of transplanted cells, which to them indicated that each focus might represent the progeny of a single ARC. This reasoning might still be valid as far as the B-lymphocyte ARC line is concerned, but the AFC which is formed in response to "thymus-dependent" antigen, obviously do not represent the progeny of a single ARC. In the Roitt et al. model these cells are the product of T-lymphocyte stimulation upon a B-lymphocyte. Probably, they explain, the T-lymphocyte binds antigen at its surface and stimulates a large number of B-lymphocytes through cell-to-

cell contact. This means therefore, that counts of foci of antibody activity in spleen cell suspensions cannot be correlated with an ARC population. Nevertheless, if AFC foci are detected in response to "thymus-dependent" antigenic challenge, then adequate numbers of both T and B-lymphocytes must have been present.

If, therefore, an experiment was designed to evaluate the effect of erythropoietic stimulation upon an ARC population, then the AFC response might still, though indirectly, reflect ARCs.

It has been shown by Abdou et al. (95) that following administration of antigen (SRBC) there was a selective and specific loss of the bone marrow's ability to provide ARC to that antigen. This loss was not evident until four hours after antigen administration but persisted for more than twenty-four hours. They postulate that ARC emigrate from the bone marrow in response to antigen injection. Since many antigens, among them being SRBC, increase the numbers of spleen colonies, then it might be postulated that antigen stimulates the hemopoietic precursor stem cell to divide into both lymphopoietic stem cells and CFU through its effect of depleting the bone marrow store of ARC. On the other hand, since antigen stimulates lymphocyte proliferation in the spleen, it is possible that its effect on spleen colonies is related to an effect upon the microenvironment of the spleen.

If bleeding stimulates all the elements of the hemopoietic system, then it might do so by stimulating release of a number of "poietins", i.e. erythropoietin, granulopoietin, megakaryocytopoietin, and perhaps lymphopoietin. It does stimulate release of erythropoietin,

but the others have not yet been identified. An alternative explanation is that bleeding stimulates the common precursor stem cell, the response of which is to increase proliferation and differentiation of all the cells of the hemopoietic system in their usual ratios.

On the other hand, if bleeding selectively stimulates hemopoietic stem cells to differentiate along erythropoietic lines, or generates erythropoietin only, then bone marrow from a pre-bled recipient could be expected to contain more cells committed to erythropoiesis and fewer cells capable of replenishing the other cell pools. Since Schooley et al. (78) have reported that new CFU are not formed until erythropoiesis is approaching maximal levels, it is reasonable to expect that bone marrow from bled mice might better support erythropoiesis at the expense of AFC or CFU when it was used to regenerate the marrow of a lethally radiated mouse.

In order to examine a possible competitive influence between the thymus-dependent immune system and the erythropoietic system, it would be desirable to assay CFU, ARC, and Fe^{59} uptake in the same animal and at the same time, after either an antigenic or erythropoietic challenge. Aside from the technical problems of assaying all these in a group of animals at one time, there is a more fundamental problem, in that the stem cells in the hemopoietic system differentiate rapidly, recovery is observable within two days, and replacement is essentially complete within fourteen days. Twelve days after bone marrow injection is usually too late to perform an accurate CFU assay because exogenous colonies are becoming confluent; the immune system does not begin

however, to recover for ten days, and normal numbers of ARC are not present until twenty-five days, (94). The lag in recovery of the immune response is related to the recovery of the thymus after heavy irradiation.

The next experiment was designed to apply a competitive stimulus to a precursor cell hypothesized to be common both to the immune system and to the hemopoietic system.

It was reasoned that bleeding a mouse might result in proliferation of the erythropoietic cells at the expense of lymphopoietic cells if bleeding exerted a competitive stimulus upon the stem cell. If bone marrow from bled animals was removed while it was still under maximal erythropoietic stimulation and injected into a lethally irradiated host, then the host upon recovery might demonstrate increased erythropoiesis and diminished AFC when compared to a control mouse.

On the other hand, bone marrow from a mouse treated with antigen but not pre-bled might not support erythropoiesis when injected into a lethally irradiated host as well as bone marrow from control mice.

Nine week old, male C3H mice were separated into three groups (Fig. 6). Five mice from one group were bled 0.5 ml. from the tail to provide maximum erythropoietic stimulation. Six hours later they were challenged with an I.V. injection of 1×10^8 sheep red blood cells (SRBC) to provide maximum antigen stimulation. Sixteen hours later, during which interval they had time to deplete existing ARC and

Figure 6: Design of Experiment to Study the Effect of Bleeding and SRBC Upon Erythropoietic and Immunologic Recovery and Exogenous Spleen Colony Formation

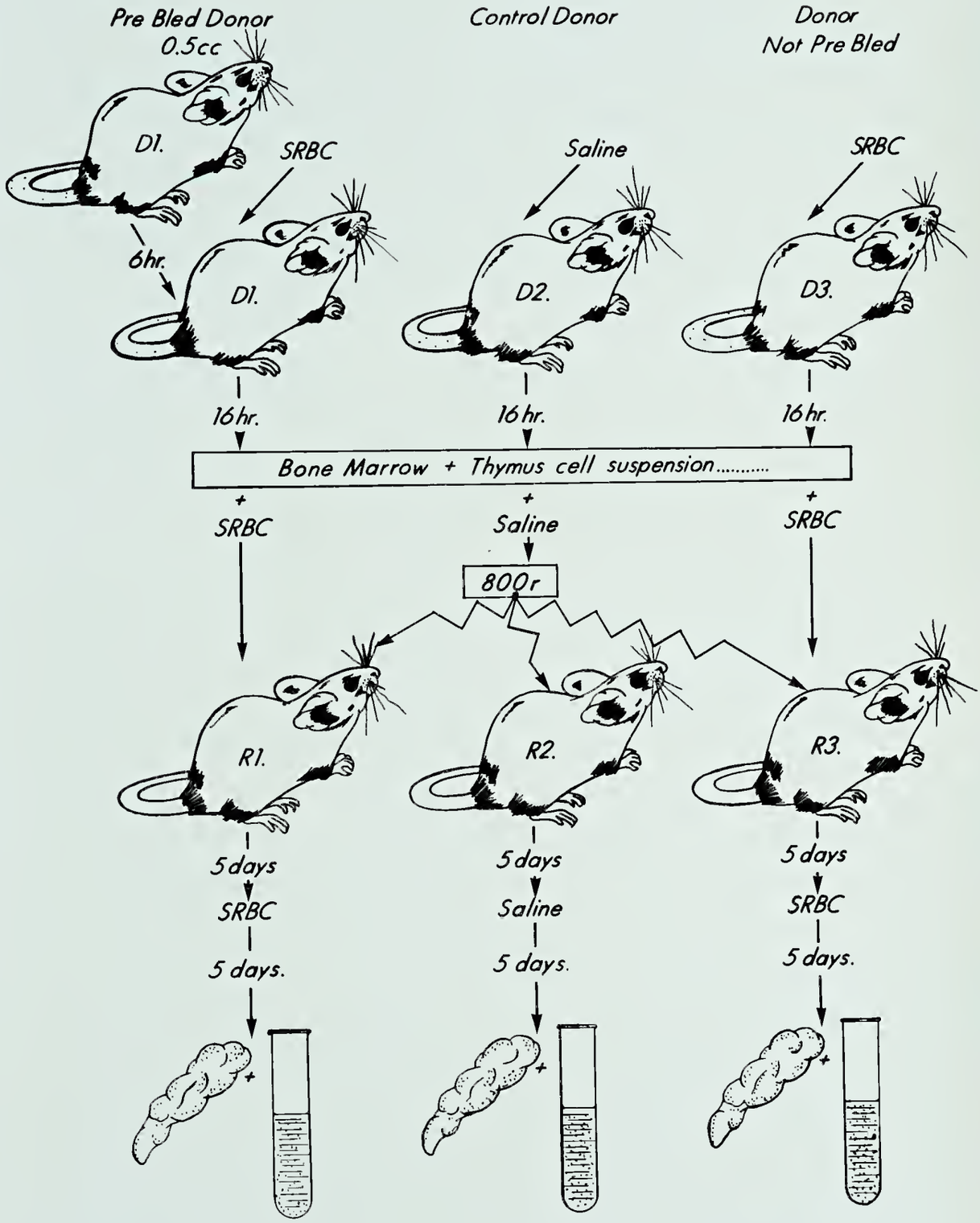
The first donor group of mice are bled 0.5 cc. and then 6 hours later challenged with 1×10^8 sheep red blood cells (SRBC) I.V. 16 hours later these mice are used as donors for 1×10^5 mixed femoral and tibial bone marrow cells and 1×10^5 thymus cells which are injected with 1×10^7 SRBC into the lethally irradiated recipient mice of the same age and strain.

The second donor group of mice are not bled or challenged with SRBC. They are used as donors for 1×10^5 mixed femoral and tibial bone marrow cells and 1×10^5 thymus cells which are injected with N. saline into the lethally irradiated recipient mice of the same age and strain.

The third donor group of mice are not bled but are challenged with 1×10^8 SRBC I.V. and 16 hours later these mice are used as donors for 1×10^5 mixed femoral and tibial bone marrow cells and 1×10^5 thymus cells which are injected with 1×10^7 SRBC into the lethally irradiated recipient mice of the same age and strain.

Five days after the bone marrow graft, all recipient mice except the control group are rechallenged with SRBC.

Eleven days after bone marrow grafting, all recipient mice are subjected to erythropoietic and spleen colony studies and AFC assay.


Pre Bled Donor
0.5cc
Control Donor
Donor
Not Pre Bled
D1.
SRBC
Saline
SRBC
6hr.
D1.
D2.
D3.
16hr.
16hr.
16hr.
Bone Marrow + Thymus cell suspension.....
+
SRBC
+
Saline
+
SRBC
800r
R1.
R2.
R3.
5 days
5 days
5 days
SRBC
Saline
SRBC
5 days.
5 days.
5 days.

presumably stimulate the bone marrow stem cell to initiate regeneration of their numbers (90), they were used as bone marrow and thymus cell donors for twelve lethally radiated (800 r) syngeneic recipients of the same age. The erythropoietic stimulus which resulted from this degree of bleeding is expected to be maximal for more than the twenty-two hour interval between bleeding and donation of marrow cells. The lethal radiation received by the recipient and low dose of transfused cells was expected to maintain erythropoiesis at a maximum for the ensuing ten day test period. To maintain maximal antigenic stimulation in the recipient, 1×10^7 SRBC was injected in suspension with the 5×10^5 bone marrow and 5×10^5 thymus cells, and an additional booster injection of 1×10^7 SRBC was given five days later. A second group of five mice was treated in an identical fashion except they were not pre-bled. A third group was neither pre-bled nor injected with antigen, but received an equivalent volume of normal saline instead of SRBC. The thymus cell suspensions were administered because the sheep erythrocyte-antigen reactive cells can not be formed from the precursor cells of the bone marrow in the absence of thymus cells. In a lethally irradiated host AFC to sheep erythrocytes are not formed unless thymus cells are administered with bone marrow cells (3).

Ten days after bone marrow transplantation, CFU and AFC were assayed by counting fresh spleen colonies and AFC. Hematocrit, reticulocyte count, serum radioiron, rbc, spleen, and femur Fe^{59} uptake, and spleen weight were determined as in previous experiments.

Because of the complexity of the techniques, half of each group

of animals was treated on each of two successive days, and relatively small numbers of animals were used.

AFCs were assayed by the plaque-forming cell assay devised by Cunningham (66).

It was reasoned, that if CFU compete with lymphopoietic stem cells for a common precursor, then a mouse challenged by bleeding might initially commit its bone marrow stem cells to proliferate along hemopoietic lines at the expense of lymphopoietic lines. Bone marrow from this bled mouse might then better support erythropoiesis at the expense of AFC when it was used to regenerate the bone marrow of a lethally irradiated isologous host, compared to normal bone marrow grafted into a lethally irradiated isogeneic host. The results of this study are listed in Table 12.

Similarily, if ARCs compete with the CFU for a common precursor stem cell, then bone marrow from a mouse challenged with SRBC antigen might better support an immune response at the expense of erythropoiesis when it was used to regenerate the marrow of a lethally irradiated isogeneic host. The results of this study are listed in Table 13.

If there is competition for the common precursor stem cell, then comparing the two experimental groups discussed above might reveal that bone marrow from pre-bled and antigen challenged mice (PB) would better support erythropoiesis than an immune response as compared to bone marrow from mice not pre-bled but antigen challenged (NPB) only,

TABLE 12: Erythropoietic and Immunologic Recovery and Exogenous Spleen Colony Formation in Mice Which Were Recipients of Bone Marrow from Isogeneic Donors Challenged by Bleeding and With Antigen (PB), Compared to Recipients of Normal Bone Marrow (Control)

GROUP		N	MEAN	S.D.	P
PB	Hematocrit	6	28.8	2.85	
Control		6	28.5	1.50	
PB	% Reticulocyte	6	1.20	1.12	<0.005
Control	Count	6	0.90	0.13	
PB	Serum Fe^{59}	5	15.97	1.79	<0.005
Control	Content	6	10.61	1.78	
PB	RBC Fe^{59}	5	4.78	2.79	<0.05
Control	Uptake	6	8.69	1.58	
PB	Exogenous Fresh	6	17.0	3.27	
Control	Spleen Colonies	6	15.8	1.86	
PB	Spleen	7	74.87	0.96	<0.005
Control	Weight	7	101.90	1.46	
PB	Spleen Fe^{59}	5	5.74	0.96	<0.005
Control	Uptake	6	8.74	1.46	
PB	Femur Fe^{59}	5	1.12	0.34	
Control	Uptake	6	1.24	0.19	
PB	AFC/10^6 Spleen	6	8.30	7.37	
Control	Cells	6	1.55	0.55	
PB	Spleen Cells x	6	61.12	4.62	<0.02
Control	10^6 in 1 ml.	6	89.67	22.19	

TABLE 13: Erythropoietic and Immunologic Recovery and Exogenous Spleen Colony Formation in Mice Which Were Recipients of Bone Marrow from Isogeneic Donors Challenged with Antigen Only (NPB) Compared to Recipients of Normal Bone Marrow (Control)

GROUP		N	MEAN	S.D.	P
NPB	Hematocrit	9	29.7	3.16	
Control		6	28.5	1.50	
NPB	% Reticulocyte	10	1.05	0.19	
Control	Count	6	0.90	0.13	
NPB	Serum Fe^{59}	9	10.41	1.87	
Control	Content	6	10.67	1.78	
NPB	RBC Fe^{59}	9	6.83	1.36	<0.05
Control	Uptake	6	8.69	1.58	
NPB	Exogenous Fresh	10	18.7	2.61	<0.05
Control	Spleen Colonies	6	15.8	1.86	
NPB	Spleen	10	99.34	9.18	
Control	Weight	7	101.90	10.90	
NPB	Spleen Fe^{59}	10	8.57	1.33	
Control	Uptake	6	8.74	1.46	
NPB	Femur Fe^{59}	10	1.27	0.18	
Control	Uptake	6	1.24	0.19	
NPB	AFC/10^6 Spleen	10	21.14	21.70	
Control	Cells	6	1.55	0.55	
NPB	Spleen Cells x	10	82.60	11.04	
Control	10^6 in 1 ml.	6	89.67	22.19	

TABLE 14: Erythropoietic and Immunologic Recovery and Exogenous Spleen Colony Formation in Mice Which Were Recipients of Bone Marrow from Isogeneic Donors Challenged by Bleeding and with Antigen (PB) Compared to Recipients of Bone Marrow Challenged with Antigen Only (NPB)

GROUP		N	MEAN	S.D.	P
PB	Hematocrit	6	28.8	2.85	
NPB		9	29.7	3.16	
PB	% Reticulocyte	6	1.20	1.12	
NPB	Count	10	1.05	0.19	
PB	Serum Fe^{59}	5	15.97	1.79	<0.001
NPB	Content	9	10.41	1.87	
PB	RBC Fe^{59}	5	4.78	2.79	
NPB	Uptake	9	6.83	1.36	
PB	Exogenous Fresh	6	17.0	3.27	
NPB	Spleen Colonies	10	18.7	2.61	
PB	Spleen	7	74.87	13.50	<0.005
NPB	Weight	10	99.34	9.18	
PB	Spleen Fe^{59}	5	5.74	0.96	<0.001
NPB	Uptake	10	8.57	1.46	
PB	Femur Fe^{59}	6	1.12	0.34	
NPB	Uptake	10	1.27	0.18	
PB	AFC/10^6	6	8.30	7.37	
NPB	Cells	10	21.14	21.70	
PB	Spleen Cells x	6	61.12	4.62	<0.001
NPB	10^6 in 1 ml.	10	82.60	11.04	

which might better support an immune response than erythropoiesis. The results of this comparison are listed in Table 14.

The AFC assay in some ways confuses the results and will therefore be dealt with first. It might appear from Table 12 that bone marrow from pre-bled and antigen treated mice supported no more AFC in their recipient hosts (despite the fact that the hosts had two booster doses of antigen) than the background numbers of AFC formed in hosts which had received no antigen and normal bone marrow. This cannot be concluded for the following reasons: firstly, the number of AFC/10^6 spleen cells ranged in the PB mice from 6.7 to 21.1 except for two mice which produced 0.3 and 0.6 AFC respectively, and in the control mice from 0.4 to 2.0. The background numbers of AFC were therefore low and reasonably constant, and four mice in the PB group obviously produced higher than background numbers of AFC. Secondly, for statistical accuracy it is estimated that there should be at least thirty-five AFC foci per slide (97) and this was achieved on only one slide in the PB group. It can only be concluded therefore, that some PB mice were capable of forming AFC.

The concentration of spleen cells used per slide ranged from 3 - 5 x 10^6 cells per 0.05 ml. and stronger concentrations were not used because pilot studies indicated that this dose would be adequate without producing excessive or confluent numbers of AFC. In Table 13 again, mice primed with antigen did not have significantly more AFC than background controls, yet the numbers of AFC in the NPB group ranged from 3.0 to 65.4/10^6 cells. In this case however, adjusting the figures by taking the square roots fits them into a normal distribution, and the mean of

the resulting non-skewed curve is significantly higher than the adjusted figures of the control group (Table 13, Appendix). The AFC assay results in Table 14, as in Table 12, do not provide statistical evidence that the PB group differs from the NPB group.

It can be concluded from the AFC assay then, that anaemic mice (as judged from the hematocrit levels) which are presumably under moderate to maximal erythropoietic stimulation, can still regenerate AFC. Furthermore, they do this in spite of Abdou's evidence which indicates that there were no ARC in their original bone marrow graft because this graft came from an immunized animal. If the ARCs were not produced by the donor marrow, then for the AFC response to have occured, ARCs must have been present in the recipients bone marrow, spleen or lymph nodes (99). It has been shown however, that while AFCs may be radioresistant to 800 r, ARCs are radiosensitive (99), therefore the ARCs were probably of donor bone marrow origin. That there may be erythropoietic and lymphopoietic competition for a common precursor stem cell cannot be supported on the basis of this assay in these experiments. Unfortunately, because the numbers of AFC obtained was fewer than were required for statistical significance, the converse cannot be presumed either.

The results listed in Table 12 reveal that bone marrow from pre-bled and antigen challenged mice behaves significantly differently from normal bone marrow. The experimental bone marrow appeared to be unable to repopulate the cells of the spleen to the same degree as normal bone marrow. The spleens in the experimental animals were significantly lighter, contained fewer cells, and took up less radioiron.

The serum radioiron content which was noted earlier to reflect unutilized radioiron, was elevated, radioiron uptake in the red cells of peripheral blood was lower, and the percentage reticulocyte count was higher in the experimental group. It cannot be concluded that the total loss of spleen cells and weight represents erythropoietic tissue loss only. As discussed before, the AFC assay did not provide a statistically useful measure of immune competence, and was not designed to quantitate lymphopoietic cells. Nevertheless, that the numbers of spleen colonies was not statistically different between experimental and control spleens, but that the spleen radioiron uptake was significantly less, suggests indeed that much of the cellular loss was erythropoietic. A significant number of colonies in the experimental group by the same reasoning, were not erythropoietic. SRBC administration has been shown to increase the numbers of spleen colonies, and referring to Table 13, it did so in this study. This opens up another avenue of speculation concerning the spleen colonies in Table 12, since the experimental group here received SRBC in identical concentrations and timing as did the experimental group in Table 13, but did not have significantly more colonies. It is possible that the bone marrow from the PB mice would have supported fewer spleen colonies than control bone marrow if SRBC had not been administered. That the increased numbers of spleen colonies in the NPB mice (Table 13) was not associated with an increase in splenic radioiron uptake, suggests that these colonies were not erythropoietic.

The only other way in which SRBC appeared to affect the study,

as seen from Table 13, is that the NPB mice had significantly less radioiron uptake in their circulating red cells, despite similar hematocrit and percentage reticulocyte counts. The reason for this is somewhat obscure but it appears to be constant, i.e. it is seen as well in the PB mice (Table 12) which were similarily treated with SRBC, but not seen when the PB and NPB mice are compared (Table 14). Since the hematocrit and percentage reticulocyte counts are not different, it is probably not an artifact caused by circulating transfused SRBC, and it is unlikely that circulating SRBC affected the erythropoietin levels. Since each experimental group mouse received 2×10^7 SRBC however, it is possible that the radioiron studies reflect iron available to the experimental animals from hemolysed cells. This observation, made in retrospect, clouds the results of this experiment.

Comparing Tables 12, 13, and 14 confirms the following points: firstly, pre-bled bone marrow results in fewer spleen cells, decreased spleen weight and radioiron uptake, increased percentage reticulocyte count, increased serum radioiron content, and possibly fewer erythropoietic spleen colonies. Secondly, SRBC administration results in increased numbers of spleen colonies which are probably not erythropoietic, and decreased red blood cell iron uptake. Thirdly, bone marrow in the recipient animals, as measured by its ability to take up radioiron, was regenerated normally whether it came from PB or NPB mice.

Marsh et al. (85) studied the effect of bleeding upon the spleens of normal mice and upon endogenous spleen colony formation in

irradiated mice. They found that normally, there is a large increase in spleen radioiron uptake and spleen weight following bleeding. Mice bled before irradiation had increased spleen weight, numbers of spleen colonies, and spleen radioiron uptake compared to not-bled controls. Bleeding before irradiation also significantly increased the survival of the irradiated mice, but the radioprotective effect was abolished by prior splenectomy. They also calculated that bled mice had a significant reduction in bone marrow CFU compared to normal mice.

Combining the results of Tables 12 and 13 with these studies, it is reasonable to assume that bleeding a mouse probably stimulates differentiation of erythroid precursors and causes a shift of CFU out of the bone marrow and into the spleen. The spleen may then become the primary site of hematopoiesis, since post irradiation deaths are generally attributed to infection and bleeding (85) but not anaemia. This implies that there is a deficiency of granulocytes, lymphocytes, and platelets in radiated animals. Bone marrow from bled mice is deficient in CFU by virtue of this shift. In Hanna's (87) studies upon the response of rat bone marrow to bleeding, he concluded that bleeding markedly increases the proliferative activity of erythropoietic precursor cells. He believes that the effect takes place within three hours after bleeding, and that bleeding exerts a direct influence upon the rate of proliferation in the progenitor cell pool rather than by controlling the differentiation of cells into recognizable erythropoietic precursors. If his model is applicable to mice, then it might be assumed that the progenitor cell pool, like the CFU, must shift out

of the bone marrow or else the bone marrow should have supported erythropoietic recovery.

The effect of antigen upon the bone marrow, that is depletion of ARC and increased numbers of CFU, might be explained if ARC depletion resulted in asymmetric division of the stem cell. One daughter cell entering a lymphopoietic committed but not necessarily an ARC line, and the other becoming a CFU. In this argument the increased numbers of CFU is the result of depleting numbers of ARCs. The converse argument that decreased numbers of ARCs are present as a result of increased numbers of CFU could be entertained. The most reasonable explanation for the latter postulate would probably be as follows, assuming that the ARC is indeed produced by a CFU in some stage of its subsequent proliferation, then the first stimulus in the regeneration of AFC or to the bone marrow by antigen would be upon the CFU.

An alternate explanation for the results could be that upon antigen stimulation and the subsequent departure of large numbers of lymphocytes from the bone marrow, that 5×10^5 bone marrow cells would be expected to contain an increased proportion of cells which did not leave the marrow, i.e. CFU. This would account for the increased numbers of spleen colonies formed when the SRBC treated marrow was exogenously assayed. Subsequent proliferation of precursor stem cells, if it produced hemopoietic precursors as well in an attempt to replace the lymphocytes, would account for the increase in total body CFU found in the endogenous assay. If bleeding caused a tremendous proliferation in

immature but committed cells of the hemopoietic lines, but did not cause proliferation of the precursor cells, and since the immature hemopoietic cells do not normally leave the bone marrow, then 5×10^5 bone marrow cells would here contain proportionately fewer precursor stem cells, and therefore proportionately fewer CFU.

CONCLUSIONS:

It is concluded, firstly, that bone marrow from pre-bled mice is deficient in its ability to restore splenic erythropoiesis, spleen weight, and probably spleen colonies to radiated hosts. Secondly, neither pre-bleeding nor SRBC antigen challenge affects the ability of the donor bone marrow to restore erythropoiesis in the host bone marrow. Thirdly, SRBC antigen increases the number of CFU in the bone marrow, but that the colonies formed are probably not erythropoietic. Finally, there was no evidence to indicate that either bleeding or the immune response competed with one another for a stem cell hypothesized to be common to both lines.

IX. DISCUSSION

1. SUMMARY OF RESULTS:

In order to study the relationship of the thymus to erythropoiesis, experiments were devised in which all the bone marrow elements were stressed by radiation, and both the colony forming units and erythropoietic recovery were compared in groups of mice half of which were thymectomized and half sham thymectomized. If the thymus produced a humoral factor which directly affected erythropoiesis or erythropoietic spleen colonies, or if contact with the thymus affected either of these, then this model should have demonstrated it. It did not, however, demonstrate any effect of the thymus upon erythropoiesis, though bone marrow, spleen, and circulating blood were all studied. Since thymic effect upon erythropoiesis had been noted with this model by other observers, it was possible that the thymus affected a population of bone marrow cells which had a different radiation sensitivity from other cells in the same marrows. Identical studies performed at radiation doses ranging from 350 r to 875 r again failed to demonstrate any effect of the thymus upon either erythropoiesis or erythropoietic spleen colonies.

Because erythropoietic recovery and erythropoietic spleen colony development has been shown to be critically affected by body levels of erythropoietin, it was questioned whether thymectomized mice might have different levels of erythropoietin than sham thymectomized controls. It was postulated that different levels of erythropoietin

might serve to counteract the effect which might otherwise be manifest between the experimental and control mice. In another experiment where thymectomized and sham thymectomized groups of mice were irradiated and half of each group subjected to bleeding, there was again no observable difference in erythropoiesis or erythropoietic spleen colonies between experimental and control groups of mice. The only erythropoietic effect of bleeding, which is normally a potent stimulator of the bone marrow, was in its predictable reduction of the hematocrit. It is argued by some observers that the bleeding stimulates the bone marrow precursor cells, including the CFU to proliferate, but that erythropoietin stimulates only the erythropoietic system and has no effect upon the CFU except for its ability to induce proliferation in prepared erythropoietic spleen colonies. It is interesting to note therefore that bleeding an animal which is already under maximal radiation-induced erythropoietin stimulation, does not further affect erythropoiesis in the marrow, spleen or peripheral blood, and does not increase the numbers of assayable CFU.

When a neonatally thymectomized mouse was regrafted with a thymus enclosed in a millipore diffusion chamber, it was observed by some observers (2) that the thymic-dependent immune response was at least partially restored, but that lymphocyte proliferation probably was not restored. This could be interpreted by postulating that the thymus produces a humoral substance responsible for immune competence, but that cellular contact is necessary for lymphocyte proliferation. If lymphocyte proliferation is induced after thymic contact only, then it is difficult to see how the thymus could effectively compete for a

bone marrow stem cell. Under normal circumstances the work on spleen colonies supports Till and McCulloch's (46) earlier belief that the bone marrow liberates a steady, regular stream of CFU, and there is no reason to postulate that the output of other bone marrow cells should be irregular. It is therefore reasonable to assume that thymic contact is responsible for lymphocyte proliferation. That administration of SRBC antigen caused evidence of bone marrow proliferation and ARC depletion within a few hours in Abdou and Richter's study (95), suggests that under abnormal circumstances at least, there might be a thymic influence upon the marrow cells. Such an effect could be mediated by a thymic humoral mechanism or possibly through the prior seeding of ARC into the marrow. If the thymus affects marrow erythropoiesis by influencing lymphocyte populations through humoral mechanisms, then the studies just discussed in this thesis would be expected to reveal that influence.

The concept that the thymus might induce immune competence through humoral mechanisms and proliferation through contact, is incompatible with the current concepts of the thymic function. It is currently believed that the role of the thymus in the immune response is to amplify a specific antigenic signal to a magnitude such that it is immunologically audible (100). This signal is carried by the thymus-dependent ARC or T-lymphocyte which does not produce antibody or differentiate into cells which do. In the presence of an intact thymus, it is believed that the T-lymphocyte does differentiate and proliferate to produce lymphs which make up the circulating pool of immune competent cells some of which have become memory cells for a specific antigen

and others have cytotoxic properties not related to antibody. Any discussion concerned with the relationship of the thymus to erythropoiesis should probably be centered around our knowledge of the thymus as it is related to this model.

It was suggested that the thymus might influence erythropoiesis through an effect of thymic-dependent lymphocytes upon the environment of the bone marrow or spleen. Since it is possible that these cells are functionally intact following thymectomy and sublethal radiation, an experiment was devised to deplete them from one group of mice and then compare post irradiation erythropoietic spleen colonies and erythropoietic recovery with a group of mice having intact thymuses. Once again, there was no observable effect of the thymus upon erythropoiesis.

The possibility remained that the thymus exerted an effect upon erythropoiesis at the stem cell level, but that this effect was not demonstrated because perhaps the assay methods were not sensitive enough to reveal it. Further, all of the studies depended upon a radiated thymus (albeit over a wide range of doses) which might have been injured in such a way as to be unable to express its erythropoietic effect, or because the thymic-dependent immune response was not specifically challenged. If competition for a common stem cell occurred between the thymic-dependent immune response and erythropoietic recovery, then increased immune reactivity and decreased erythropoietic activity might be seen in an antigen treated group of mice, and the converse in a bled group of mice. Once again, an experiment was performed to test this

hypothesis, but the thymus, as reflected through an immune response, did not appear to influence erythropoiesis, and bleeding probably did not affect the immune response.

2. THE THYMUS AND ERYTHROPOIESIS:

The results of the studies presented in this thesis leads the author to seriously question whether the thymus has any effect upon erythropoiesis. In the study reported by Tunis et al. (44), some of their neonatally thymectomized groups of mice developed a normoblastic hypoplasia and anemia without evidence of either wasting disease or Coomb's positive hemolytic anemia. They postulated that the anemia was probably secondary to infection and/or anti-erythropoietic antibodies, and concluded that it was certainly related to immunological deficiencies in the immature mouse. Dameshek et al. (21) also concludes that autoimmunity is the most likely cause of anemia when it is associated with thymoma in humans. Pure red cell anemia is felt by most observers to have multiple causes (101), and the occurrence of plasma inhibitors and antinuclear factors in patients with thymoma and pure red cell anemia is described (102) (103). The development of an immunoassay for erythropoietin may settle the controversy (104).

In the introduction to this thesis a number of investigations were summarized which led the different authors to conclude that the thymus might be a site of erythropoiesis, that the thymus might induce or support erythropoiesis in the spleen, and that the thymus might suppress erythropoiesis in the spleen. With the exception of the study by

Goodman and Shinpock (32) who measured radioiron incorporation, all of these studies evaluated erythropoiesis on morphological grounds. The study reported by Campbell et al. (30) failed to demonstrate any erythropoiesis in the mouse thymus or any erythropoietic effect of the mouse thymus on the evidence of radioiron uptake or autoradiography. Though the experiments reported in this thesis were not designed to test any of these studies, it is possible that the morphologic criteria used to differentiate primitive cells in solid organs by these authors gave misleading results. The lack of splenomegally in their recipient mice led Goodman and Shinpock to conclude that graft versus host reaction (GVHR) had not caused increased splenic erythropoiesis in their thymus treated mice. The experimental animals in their study were lethally radiated F_1 hybrids which had received parental bone marrow and thymus cells. This combination would be expected to cause GVHR and Pinno and Bain (45) have reported a marked shift of erythropoiesis from the bone marrow to the spleen in the GVHR.

No explanation can be provided for the differences between the results of Globerson and Feldman (13) and the studies reported in this thesis. They reported increased numbers of erythropoietic spleen colonies in thymectomized mice which had received 550 r ten days earlier, and that there were equal numbers of colonies in the thymectomized and control mice but still an increased erythropoietic population in the thymectomized spleens which were studied at eleven days. They found that thymectomized mice developed an average of one new colony but that control mice developed an average of seven

new colonies between the tenth and eleventh day. The 550 r studies reported in this thesis were all assayed on the eleventh day and there was no erythropoietic or erythropoietic spleen colony difference observable between experimental and control mice. Because of the different post irradiation times of assaying spleen colonies, the experiments can not be considered to be contradictory.

Numerous investigators have cultured thymus, and thymus cell suspensions in millipore diffusion chambers in isogeneic hosts, but Albert et al. (28) appear to be the only investigators who have specifically studied erythropoiesis. Cimo and Walker (105) listed the principal cell types found within millipore diffusion chambers in which explants of neonatal or adult thymus cells were cultured from four to thirty-one days. They do not include any erythropoietic cells in their list, and point out that the only thymus cell transformations they recognized were cells with bizarre and giant nuclei or multinucleate cells. Their control groups included spleen explants and empty explants. No erythropoietic cells are listed as being found in spleen millipore chambers, and no cells were found in the empty chambers. If eighteen percent of the cells had belonged to erythropoietic lines, presumably they would have included this result.

3. SPECULATIONS CONCERNING NON-ERYTHROID SPLEEN COLONIES:

It was earlier noted (Section V., Page 52) that following a lethal dose of radiation, the numbers of deaths were much increased in the thymectomized compared to the sham thymectomized mice over the

same length of time. Spleen colonies were not counted in these mice. A similar high mortality was later noted in thymectomized lethally irradiated mice compared to sham thymectomized lethally irradiated mice after both groups had been replaced with identical doses of bone marrow cells (Section VII., Page 71). Table 15 (Appendix) notes the mean numbers of exogenous spleen colonies which were found in the mice which died. Since post irradiation survival correlates well with numbers of spleen colonies, it is not surprising that there was a higher percentage of dead mice having fewer colonies. Of the group that was subsequently found to have a mean of less than two hemopoietic spleen colonies, eighty-two percent of thymectomized group mice had died, while of the thymectomized group which was found to have a mean of more than nine spleen colonies, only sixty-two percent had died. For the control mice, sixty-three percent of the group having a mean of less than two spleen colonies had died, but only twenty-one percent of the group with a mean of eleven spleen colonies had died. This data supports the observation that post irradiation survival is related to the numbers of spleen colonies.

Table 15 (Appendix) also reveals that despite the same numbers of spleen colonies found in the dead mice, a higher percentage of thymectomized mice than sham thymectomized mice died. Thymectomized adult irradiated mice develop a wasting disease which is attributed to loss of immune competence and resulting infection. It is unlikely that the thymus would have a radioprotective effect which was independent of immune competence.

Hanks and Ainsworth (106) studied the correlation between

post irradiation survival and CFU by treating mice with endotoxin to increase the numbers of spleen colonies. They found that in their model, there was no consistent correlation between survival and numbers of CFU. This is the same conclusion that Marsh et al. (85) came to when they increased the numbers of spleen colonies by administration of erythropoietin. They attribute post irradiation death to a reduction of granulocytes and/or platelets. They note furthermore, that the radioprotective effect of pre-irradiation bleeding is abolished by splenectomy which implies that the spleen in irradiated animals contributes significant numbers of granulocytes and platelets to the circulation. Fred and Smith (107) noted that weanling mice had a lower percentage of post irradiation survival than adult mice and the weanlings also had lower post irradiation granulocyte counts.

It was noted in Section V., Tables 3 and 4 of this thesis, that there were fewer spleen colonies in thymectomized mice compared to control mice after 550 r. Since the spleen radioiron uptakes were equal, these appeared to be non-erythropoietic spleen colonies. This finding was interpreted with caution since it was not reflected by the other methods used to estimate numbers of spleen colonies (Appendix Tables 3 and 4), and was abolished by post irradiation bleeding (Table 9).

In Section VIII., Table 13, SRBC administration was noted to have caused an increase in the numbers of spleen colonies, and once again on the basis of the splenic radioiron uptake, these appear to be non-erythropoietic colonies. Since megakaryocyte colonies do not

form bulges on the surface of the spleen and are therefore not countable by the assay techniques used in this thesis, it follows that non-erythroid colonies are probably granulocytic. It is possible therefore that erythropoietin and perhaps endotoxin preferentially stimulate erythropoietic differentiation over granulopoietic differentiation in spleen colonies, and that the thymic-dependent SRBC antigen preferentially stimulates granulopoietic differentiation over erythropoietic differentiation in spleen colonies. If bleeding directly stimulates the bone marrow precursors to form more CFU and stimulates erythropoiesis only through the release of erythropoietin, then it would be expected to have better irradiation protection than erythropoietin alone. If the thymus has any effect upon granulopoiesis, then administration of SRBC antigen to a thymectomized irradiated mouse would not be expected to increase the numbers of spleen colonies. This experiment could be performed in plethoric animals to further delineate possible non-erythropoietic colonies.

The experiments reported in this thesis were not designed to measure granulopoiesis and it has been emphasized that the decrease in spleen colonies noted in thymectomized mice after 550 r might have no biological significance. The foregoing discussion is an attempt to correlate post irradiation survival in thymectomized mice with a possible thymic effect upon granulopoiesis and it is highly speculative.

X. CONCLUSIONS

A series of experiments was designed to test the hypothesis that the thymus could influence erythropoiesis.

It was concluded that prior thymectomy had no influence upon erythropoiesis or erythropoietic spleen colonies in a post irradiation recovery period. It was further concluded that the failure to demonstrate a thymic influence upon erythropoiesis was not related to the dose of irradiation administered, to the degree of immune competence of the experimental animal, or to the degree of anemia of the experimental animals.

An experiment was designed to influence the path of differentiation of a stem cell hypothesized to be common to both erythropoietic and lymphopoietic lines. Neither bleeding nor an immune response could be shown to exert a competitive demand upon such a cell.

Other conclusions are as follows:

1. The anemia which results from 550 r induces maximal erythropoietic stimulation.
2. Post irradiation bleeding of a mouse which has been subjected to 550 r will not further stimulate erythropoiesis.
3. There are significantly fewer endogenous spleen colonies in thymectomized mice recovering from 550 r and it is speculated that these represent non-erythropoietic colonies. The biological significance of this

result is questioned.

4. There are significantly fewer endogenous spleen colonies in normal but not in thymectomized mice which are subjected to bleeding in the post irradiation recovery period. The loss appears to be of non-erythropoietic colonies and the biological significance of this result is also questioned.
5. Bone marrow taken from mice which have been bled is deficient in its ability to restore splenic erythropoiesis, spleen weight, and probably spleen colonies in radiated hosts.
6. Neither pre-bleeding nor a sheep red blood cell antigen challenge to a donor mouse affects the ability of its bone marrow to restore erythropoiesis in the bone marrow of a radiated host.
7. Sheep red blood cell antigen increases the numbers of colony forming units in the bone marrow, but the colonies that are formed are probably not erythropoietic.

XI. APPENDIX

1. STATISTICAL ANALYSIS

The difference between the means of experimental and control groups were analysed by utilizing the unpaired t-test (98).

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{(X_i - X_1)^2 + (X_{2i} - X_2)^2}{N_1 + N_2 - 2}\left(\frac{1}{N_1} + \frac{1}{N_2}\right)}}$$

N_1 = size of sample$_1$

N_2 = size of sample$_2$

Degrees of freedom = $N_1 + N_2 - 2$

The differences between "fresh" and "projected" methods of counting spleen colonies were analysed by utilizing the paired t-test (98).

$$t = \frac{d^2}{\sqrt{\frac{(d_i - \bar{d})^2}{N\,(N - 1)}}}$$

Degrees of freedom = N - 1

TABLE 1: Erythropoietic Recovery and Endogenous Spleen Colonies in Thymectomized and Sham Thymectomized Mice After 350 r

GROUP		N	MEAN	S.D.	P	ADJUSTED MEAN	ADJUSTED S.D.	ADJUSTED P
Thym.	Hct.	5	36.8	1.94		6.06	0.16	
Control		5	36.4	1.02		6.03	0.08	
Thym.	% Retic.	5	1.3	0.06		1.14	0.03	
Control	Count	5	1.3	0.09		1.14	0.04	
Thym.	Serum Fe^{59}	5	8.42	5.57		2.78	0.83	
Control	Content	5	9.30	1.78		3.04	0.29	
Thym.	RBC Fe^{59}	5	13.22	4.83		3.55	0.80	
Control	Uptake	5	12.88	3.54		3.55	0.51	
Thym.	Projected Spleen	14	7.7	2.46		2.73	0.49	
Control	Colonies	15	7.9	2.79		2.77	0.50	
Thym.	Spleen	14	61.2	9.59		7.80	0.62	
Control	Weight	15	58.6	9.05		7.63	0.58	
Thym.	Spleen Fe^{59}	5	6.3	3.41		2.41	0.71	
Control	Uptake	5	5.6	0.99		2.36	0.22	
Thym.	Femur Fe^{59}	5	1.36	0.29		1.16	0.12	
Control	Uptake	5	1.48	0.20		1.12	0.09	

TABLE 2: Erythropoietic Recovery and Endogenous Spleen Colonies in Thymectomized and Sham Thymectomized Mice After 450 r

GROUP		N	MEAN	S.D.	P	ADJUSTED MEAN	ADJUSTED S.D.	ADJUSTED P
Thym.	Hct.	5	32.8	0.75		5.73	0.06	
Control		5	31.6	4.22		5.61	0.39	
Thym.	% Retic.	5	1.08	0.07		1.18	0.16	
Control	Count	5	1.22	0.08		1.50	1.19	
Thym.	Serum Fe^{59}	5	16.54	4.70		4.02	0.62	
Control	Content	5	17.10	6.52		4.06	0.79	
Thym.	RBC Fe^{59}	5	7.90	3.00		2.75	0.57	
Control	Uptake	5	6.04	4.16		2.28	0.92	
Thym.	Projected Spleen	14	6.14	1.96		2.44	0.42	
Control	Colonies	15	6.53	2.53		2.50	0.53	
Thym.	Spleen	14	44.44	6.45		6.65	0.49	
Control	Weight	15	43.21	8.74		6.54	0.68	
Thym.	Spleen Fe^{59}	5	4.28	1.39		2.04	0.33	
Control	Uptake	5	3.76	2.04		1.86	0.56	
Thym.	Femur Fe^{59}	5	1.14	0.44		1.04	0.23	
Control	Uptake	5	1.24	0.35		1.10	0.16	

TABLE 3: Erythropoietic Recovery and Endogenous Spleen Colonies in Thymectomized and Sham Thymectomized Mice After 550 r

GROUP		N	MEAN	S.D.	P	ADJUSTED MEAN	ADJUSTED S.D.	ADJUSTED P
Thym.	Hct.	4	26.8	2.16		5.17	0.21	
Control		5	24.2	2.48		4.91	0.26	
Thym.	% Retic.	5	1.05	0.09		1.12	1.19	
Control	Count	5	0.99	0.09		0.98	0.17	
Thym.	Serum Fe^{59}	5	20.50	2.73		4.52	0.31	
Control	Content	4	24.95	2.40		4.99	0.24	
Thym.	RBC Fe^{59}	5	1.66	1.15		1.13	0.62	
Control	Uptake	5	1.26	2.23		1.18	0.69	
Thym.	Projected Spleen	14	3.71	1.58	<0.01	1.87	0.45	<0.01
Control	Colonies	13	6.38	2.65		2.47	0.53	
Thym.	Spleen	14	39.04	6.40		6.23	0.51	
Control	Weight	14	39.27	6.81		6.24	0.54	
Thym.	Spleen Fe^{59}	5	3.12	1.29		1.73	0.36	
Control	Uptake	4	2.58	1.23		1.56	0.38	
Thym.	Femur Fe^{59}	5	0.91	0.26		0.95	0.14	
Control	Uptake	5	0.69	0.57		0.74	0.38	

TABLE 4: Erythropoietic Recovery and Endogenous Spleen Colonies in Thymectomized and Sham Thymectomized Mice After 550 r

GROUP		N	MEAN	S.D.	P	ADJUSTED MEAN	ADJUSTED S.D.	ADJUSTED P
Thym.	Hct.	10	33.1	2.67		5.75	0.23	
Control		10	35.6	2.82		5.97	0.24	
Thym.	% Retic	10	1.31	0.56		1.12	0.23	
Control	Count	10	1.45	0.52		1.18	0.22	
Thym.	Serum Fe^{59}	10	13.84	3.34		3.68	0.52	
Control	Content	10	15.85	8.86		3.82	1.12	
Thym.	RBC Fe^{59}	10	4.85	6.23		1.37	1.85	
Control	Uptake	9	5.47	12.04		1.41	2.93	
Thym.	Projected Spleen	10	7.60	2.10	<0.025	2.73	0.37	<0.025
Control	Colonies	10	10.70	3.16		3.24	0.47	
Thym.	Fresh Spleen	10	7.80	4.56		2.68	0.79	
Control	Colonies	10	9.10	3.88		2.91	0.78	
Thym.	Spleen Micro	10	29.50	7.14		5.39	0.65	
Control	Colonies	10	30.5	9.80		5.45	0.87	
Thym.	Spleen	10	39.71	6.19		6.28	0.46	
Control	Weight	10	45.16	6.02		6.70	0.47	
Thym.	Spleen Fe^{59}	10	3.84	3.73		1.72	0.93	
Control	Uptake	10	5.55	3.08		2.23	0.74	
Thym.	Femur Fe^{59}	10	1.16	0.85		0.89	0.45	
Control	Uptake	10	1.36	0.49		1.14	0.23	

TABLE 5: Erythropoietic Recovery and Endogenous Spleen Colonies in Thymectomized and Sham Thymectomized Mice After 600 r

GROUP		N	MEAN	S.D.	P	ADJUSTED MEAN	ADJUSTED S.D.	ADJUSTED P
Thym.	Hct.	17	29.18	3.45		5.39	0.31	
Control		16	30.12	2.45		5.48	0.22	
Thym.	% Retic.	18	1.61	0.59		1.24	0.26	
Control	Count	15	1.87	0.66		1.35	0.22	
Thym.	Serum Fe^{59}	16	18.80	6.12		4.27	0.73	
Control	Content	16	16.58	4.18		4.04	0.53	
Thym.	RBC Fe^{59}	16	6.98	6.33		2.19	1.57	
Control	Uptake	16	8.64	4.29		2.83	0.80	
Thym.	Projected Spleen	17	6.65	3.16		2.50	0.52	
Control	Colonies	16	8.19	2.70		2.81	0.63	
Thym.	Spleen	17	34.05	7.89		5.80	0.66	
Control	Weight	16	36.15	5.49		5.60	0.44	
Thym.	Spleen Fe^{59}	17	6.65	3.16		1.87	0.46	
Control	Uptake	16	8.19	2.70		2.06	0.45	
Thym.	Femur Fe^{59}	17	1.37	0.41				
Control	Uptake	16	1.39	0.35				

TABLE 6: Erythropoietic Recovery and Endogenous Spleen Colonies in Thymectomized and Sham Thymectomized Mice After 875 r

GROUP		N	MEAN	S.D.	P	ADJUSTED MEAN	ADJUSTED S.D.	ADJUSTED P
Thym.	Hct.	4	15.8	4.87		3.92	0.61	
Control		6	13.3	2.36		3.64	0.32	
Thym.	% Retic.	5	6.9	4.69		2.41	1.05	
Control	Count	6	3.6	0.90		1.87	0.24	
Thym.	Serum Fe^{59}	4	7.89	3.07	<0.001	2.76	0.51	<0.001
Control	Content	6	30.60	8.64		5.47	0.83	
Thym.	RBC Fe^{59}	4	-0.32	0.45	<0.01	-0.44	0.35	<0.01
Control	Uptake	6	-2.28	1.11		-1.44	0.46	
Thym.	Projected Spleen	5	1.6	0.49		1.25	0.20	
Control	Colonies	6	2.0	1.41		1.24	0.67	
Thym.	Spleen	5	29.28	4.73		5.39	0.44	
Control	Weight	6	24.50	3.14		4.94	0.33	
Thym.	Spleen Fe^{59}	4	0.06	0.04		0.23	0.08	<0.02
Control	Uptake	6	0.50	0.47		0.64	0.29	
Thym.	Femur Fe^{59}	4	0.06	0.03		0.22	0.07	
Control	Uptake	6	0.20	0.17		0.40	0.19	

TABLE 7: Post Radiation Erythropoietic Recovery and Spleen Colony Formation in Bled Versus Not-Bled Control Mice

GROUP		N	MEAN	S.D.	P	ADJUSTED MEAN	ADJUSTED S.D.	ADJUSTED P
Bled	Hct	7	19.1	3.74	<0.001	4.4	0.41	<0.001
Not Bled		10	35.6	2.82		6.0	0.24	
Bled	% Retic.	7	2.84	1.34	<0.05	1.64	0.37	<0.02
Not Bled	Count	10	1.45	0.52		1.18	0.22	
Bled	Serum Fe^{59}	7	18.02	4.01		4.22	0.50	
Not Bled	Content	9	15.85	8.86		3.82	1.12	
Bled	RBC Fe^{59}	7	9.91	4.80		3.04	0.81	
Not Bled	Uptake	9	5.47	12.04		1.41	2.93	
Bled	Projected Spleen	7	6.6	1.84	<0.01	2.54	0.35	<0.01
Not Bled	Colonies	10	10.7	3.16		3.24	0.47	
Bled	Fresh Spleen	7	9.14	2.90		2.99	0.46	
Not Bled	Colonies	10	9.10	3.88		2.91	0.78	
Bled	Spleen Micro	6	22.5	9.91		4.52	1.22	
Not Bled	Colonies	10	30.5	9.80		5.45	0.87	
Bled	Spleen	6	50.45	7.54		7.08	0.50	
Not Bled	Weight	10	45.16	6.02		6.70	0.46	
Bled	Spleen Fe^{59}	6	6.01	2.71		2.39	0.53	
Not Bled	Uptake	10	5.55	3.08		2.23	0.75	
Bled	Femur Fe^{59}	7	1.35	0.26		1.16	0.11	
Not Bled	Uptake	10	1.36	0.49		1.14	0.23	

TABLE 8: Post Radiation Erythropoietic Recovery and Spleen Colony Formation in Bled Versus Not-Bled Thymectomized Mice

GROUP		N	MEAN	S.D.	P	ADJUSTED MEAN	ADJUSTED S.D.	ADJUSTED P
Bled	Hct.	10	17.28	5.08	<0.001	4.11	0.59	<0.001
Not Bled		10	33.14	2.67		5.75	0.23	
Bled	% Retic.	10	2.18	1.02	<0.05	1.44	0.32	<0.05
Not Bled	Count	10	1.31	0.56		1.12	0.23	
Bled	Serum Fe^{59}	10	22.70	8.76	<0.02	4.67	0.96	<0.02
Not Bled	Content	10	13.84	3.34		3.68	0.52	
Bled	RBC Fe^{59}	10	7.62	6.99		2.24	1.75	
Not Bled	Uptake	10	6.22	4.85		1.37	1.85	
Bled	Projected Spleen	10	6.9	2.77		2.58	0.50	
Not Bled	Colonies	9	7.1	1.59		2.65	0.30	
Bled	Fresh Spleen	10	7.9	5.59		2.67	0.87	
Not Bled	Colonies	10	7.8	4.56		2.68	0.79	
Bled	Spleen Micro	10	14.40	6.53	<0.001	3.71	0.80	<0.001
Not Bled	Colonies	10	29.50	7.14		5.39	0.65	
Bled	Spleen	10	44.89	15.37		6.62	1.04	
Not Bled	Weight	10	39.71	6.19		6.28	0.46	
Bled	Spleen Fe^{59}	10	3.96	3.29		1.85	0.72	
Not Bled	Uptake	10	3.84	3.73		1.72	0.93	
Bled	Femur Fe^{59}	10	1.15	0.45		1.05	0.22	
Not Bled	Uptake	10	1.16	0.84		0.98	0.44	

TABLE 9: Post Radiation Erythropoietic Recovery and Spleen Colony Formation in Bled Thymectomized Versus Bled Control Mice

GROUP		N	MEAN	S.D.	P	ADJUSTED MEAN	ADJUSTED S.D.	ADJUSTED P
Thym.	Hct.	10	17.3	5.08		4.11	0.59	
Control		7	19.1	3.74		4.35	0.41	
Thym.	% Retic.	10	2.18	1.02		1.44	0.32	
Control	Count	7	2.84	1.34		1.64	0.37	
Thym.	Serum Fe^{59}	10	22.70	8.75		4.67	0.96	
Control	Content	7	18.02	4.01		4.22	0.50	
Thym.	RBC Fe^{59}	10	7.62	6.99		2.24	1.75	
Control	Content	7	9.91	4.80		3.04	0.81	
Thym.	Projected Spleen	10	6.90	2.77		2.58	0.50	
Control	Colonies	7	6.57	1.84		2.54	0.35	
Thym.	Fresh Spleen	10	7.9	5.59		2.67	0.87	
Control	Colonies	7	9.1	2.90		2.99	0.46	
Thym.	Spleen Micro	10	14.40	6.53		3.71	0.80	
Control	Colonies	6	22.50	9.91		4.52	1.22	
Thym.	Spleen	10	44.89	15.37		6.62	1.05	
Control	Weight	6	50.45	7.54		7.08	0.50	
Thym.	Spleen Fe^{59}	10	3.96	3.29		1.85	0.72	
Control	Uptake	6	6.01	2.71		2.39	0.53	
Thym.	Femur Fe^{59}	10	1.15	0.45		1.05	0.22	
Control	Uptake	7	1.35	0.26		1.16	0.11	

TABLE 10 : Post Radiation Erythropoietic Recovery and Spleen Colony Formation in Bone Marrow Replaced Thymectomized and Sham Thymectomized Mice

GROUP		N	MEAN	S.D.	P	ADJUSTED MEAN	ADJUSTED S.D.	ADJUSTED P
Thym.	Hct.	4	22.5	3.57		4.73	0.38	
Control		8	24.5	3.94		4.93	0.39	
Thym.	% Retic.	4	0.8	0.04		0.88	0.02	
Control	Count	8	0.9	0.18		0.9	0.09	
Thym.	Serum Fe^{59}	4	31.72	9.83		5.57	0.84	
Control	Content	8	27.54	6.81		5.20	0.70	
Thym.	RBC Fe^{59}	4	-4.72	2.14		-2.12	0.46	
Control	Uptake	8	-1.66	4.85		-0.88	9.98	
Thym.	Projected Spleen	4	4.75	2.28		2.08	0.64	
Control	Colonies	8	5.88	3.48		2.31	0.74	
Thym.	Fresh Spleen	9	8.44	4.79		2.80	0.77	
Control	Colonies	5	7.40	3.38		2.66	0.56	
Thym.	Spleen	5	27.76	4.46		5.25	0.41	
Control	Weight	9	31.31	8.54		5.54	0.78	
Thym.	Spleen Fe^{59}	4	1.12	0.16		1.56	0.80	
Control	Uptake	8	3.08	2.84		1.05	0.08	
Thym.	Femur Fe^{59}	4	0.53	0.19		0.72	0.14	
Control	Uptake	8	0.56	0.34		0.72	0.20	

TABLE 11: Post Radiation Erythropoietic Recovery and Spleen Colony Formation in Bone Marrow Replaced Thymectomized and Sham Thymectomized Mice

GROUP		N	MEAN	S.D.	P	ADJUSTED MEAN	ADJUSTED S.D.	ADJUSTED P
Thym.	Hct.	5	25.4	3.14		5.03	0.31	
Control		12	23.0	6.26		7.44	0.67	
Thym.	% Retic.	5	1.1	0.12		1.05	0.05	
Control	Count	12	1.2	0.16		1.10	0.08	
Thym.	Serum Fe^{59}	5	23.08	4.15		4.78	0.44	
Control	Content	12	25.78	8.45		5.02	0.77	
Thym.	RBC Fe^{59}	5	-0.97	2.60		-0.48	1.52	
Control	Uptake	12	-1.29	3.25		-0.57	1.56	
Thym.	Projected Spleen	5	7.60	1.85		2.74	0.34	
Control	Colonies	12	8.25	3.51		2.80	0.63	
Thym.	Fresh Spleen	6	6.67	4.11		2.44	0.85	
Control	Colonies	12	8.75	5.20		2.83	0.85	
Thym.	Spleen	6	35.99	12.78		5.71	0.49	
Control	Weight	12	32.80	5.70		5.91	1.02	
Thym.	Spleen Fe^{59}	5	2.96	1.51		1.66	0.45	
Control	Uptake	12	4.08	3.32		1.87	0.76	
Thym.	Femur Fe^{59}	5	0.77	0.26		0.86	0.16	
Control	Uptake	12	0.94	0.36		0.95	0.19	

TABLE 12: Erythropoietic and Immunologic Recovery and Exogenous Spleen Colony Formation in Mice Which Were Recipients of Bone Marrow from Isogeneic Donors Challenged by Bleeding and with Antigen (PB), Compared to Recipients of Normal Bone Marrow (Control)

GROUP		N	MEAN	S.D.	P	ADJUSTED MEAN	ADJUSTED S.D.	ADJUSTED P
PB	Hematocrit	6	28.8	2.85		5.36	0.26	
Control		6	28.5	1.50		5.34	0.14	
PB	% Retic.	6	1.20	1.12		1.09	0.05	
Control	Count	6	0.90	0.13	<0.005	0.95	0.07	<0.005
PB	Serum Fe^{59}	5	15.97	1.79		3.99	0.22	
Control	Content	6	10.61	1.78	<0.005	3.26	0.26	<0.005
PB	RBC Fe^{59}	5	4.78	2.79		2.09	0.65	
Control	Uptake	6	8.69	1.58	<0.05	2.94	0.26	<0.005
PB	Exogenous Fresh	6	17.0	3.27		4.10	0.39	
Control	Spleen Colonies	6	15.8	1.86		3.97	0.23	
PB	Spleen	7	74.87	0.96		8.62	0.78	
Control	Weight	7	101.90	1.46	<0.005	10.08	0.54	<0.005
PB	Spleen Fe^{59}	5	5.74	0.96		2.39	0.20	
Control	Uptake	6	8.74	1.46	<0.005	2.95	0.25	<0.005
PB	Femur Fe^{59}	5	1.12	0.34		1.04	0.16	
Control	Uptake	6	1.24	0.19		1.11	0.09	
PB	$AFC/10^6$ Spleen	6	8.30	7.37		2.49	1.45	
Control	Cells	6	1.55	0.55		1.22	0.27	
PB	Spleen	6	61.12	4.62		7.81	0.29	
Control	Cells x 10^6 in 1 ml.	6	89.67	22.19	<0.02	9.39	1.19	<0.02

TABLE 13: Erythropoietic and Immunologic Recovery and Exogenous Spleen Colony Formation in Mice Which Were Recipients of Bone Marrow from Isogeneic Donors Challenged with Antigen Only (NPB) Compared to Recipients of Normal Bone Marrow (Control)

GROUP		N	MEAN	S.D.	P	ADJUSTED MEAN	ADJUSTED S.D.	ADJUSTED P
NPB	Hematocrit	9	29.7	3.16		5.44	0.29	
Control		6	88.5	1.50		5.34	0.14	
NPB	% Retic.	10	1.05	0.19		1.02	0.09	
Control	Count	6	0.90	0.13		0.95	0.07	
NPB	Serum Fe^{59}	9	10.41	1.87		3.21	0.28	
Control	Content	6	10.67	1.78		3.26	0.26	
NPB	RBC Fe^{59}	9	6.83	1.36	<0.05	2.60	0.27	<0.05
Control	Uptake	6	8.69	1.58		2.94	0.26	
NPB	Exogenous Fresh	10	18.7	2.61	<0.05	4.31	0.31	<0.05
Control	Spleen Colonies	6	15.8	1.86		3.97	0.23	
NPB	Spleen	10	99.34	9.18		9.96	0.45	
Control	Weight	7	101.90	10.90		10.08	0.54	
NPB	Spleen Fe^{59}	10	8.57	1.33		2.92	0.23	
Control	Uptake	6	8.74	1.46		2.95	0.25	
NPB	Femur Fe^{59}	10	1.27	0.18		1.12	0.08	
Control	Uptake	6	1.24	0.19		1.11	0.09	
NPB	$AFC/10^6$ Spleen	10	21.14	21.70		4.10	2.08	<0.005
Control	Cells	6	1.55	0.55		1.22	0.27	
NPB	Spleen Cells x 10^6	10	82.60	11.04		9.07	0.64	
Control	in 1 ml.	6	89.67	22.19		9.39	1.19	

TABLE 14: Erythropoietic and Immunologic Recovery and Exogenous Spleen Colony Formation in Mice Which Were Recipients of Bone Marrow from Isogeneic Donors Challenged by Bleeding and with Antigen (PB) Compared to Recipients of Bone Marrow Challenged with Antigen Only (NPB)

GROUP		N	MEAN	S.D.	P	ADJUSTED MEAN	ADJUSTED S.D.	ADJUSTED P
PB	Hematocrit	6	28.8	2.85		5.36	0.26	
NPB		9	29.7	3.16		5.44	0.29	
PB	% Retic.	6	1.20	1.12		1.09	0.05	
NPB	Count	10	1.05	0.19		1.02	0.09	
PB	Serum Fe^{59}	5	15.97	1.79	<0.001	3.99	0.22	<0.001
NPB	Content	9	10.41	1.87		3.21	0.28	
PB	RBC Fe^{59}	5	4.78	2.79		2.09	0.65	
NPB	Uptake	9	6.83	1.36		2.60	0.27	
PB	Exogenous Fresh	6	17.0	3.27		4.10	0.39	
NPB	Spleen Colonies	10	18.7	2.61		4.31	0.31	
PB	Spleen	7	74.87	13.50	<0.005	8.62	0.78	<0.005
NPB	Weight	10	99.34	9.18		9.96	0.45	
PB	Spleen Fe^{59}	5	5.74	0.96	<0.001	2.39	0.20	<0.001
NPB	Uptake	10	8.57	1.46		2.92	0.23	
PB	Femur Fe^{59}	6	1.12	0.34		1.04	0.16	
NPB	Uptake	10	1.27	0.18		1.12	0.08	
PB	AFC/10^6	6	8.30	7.37		2.49	1.45	
NPB	Cells	10	21.14	21.70		4.10	2.08	
PB	Spleen Cells x 10^6	6	61.12	4.62	<0.001	7.81	0.29	<0.001
NPB	in 1 ml.	10	82.60	11.04		9.07	0.64	

TABLE 15: EXOGENOUS SPLEEN COLONIES IN THYMECTOMIZED AND SHAM THYMECTOMIZED MICE WHICH DIED

		TOTAL NUMBER WHICH DIED	MORTALITY	MEAN NUMBERS SPLEEN COLONIES	S.D.	P
GROUPS I & II	Thym.	22	82%	1.8	2.7	>0.90
	Control	17	63%	1.8	3.5	
GROUP III	Thym.	8	62%	9.2	5.0	>0.30
	Control	3	21%	11.3	1.9	

Mortality refers to the numbers of lethally irradiated (800 r) bone marrow recipient (5×10^6 cells) mice which died. Eighty percent of the mice died 5 to 12 days after bone marrow replacement and the rest died sporadically to the 28th day.

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